09/646825

532 Rec'd PCT/PTO 22 SEP 2000

Practitioner's Docket No. 55022

CHAPTER II

TRANSMITTAL LETTER
TO THE UNITED STATES ELECTED OFFICE (EO/US)
(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/JP99/01481	24 March 1999	24 March 1998
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

METHOD FOR TRANSFORMING PLANT, THE RESULTANT PLANT AND GENE THEREOF
TITLE OF INVENTION

Satoshi MORI, Hiromi NAKANISHI, Hiroyuki OKI and Hirotaka YAMAGUCHI
APPLICANTS

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

NOTE: *To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.*

WARNING: *Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. §1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing - See 37 C.F.R. §1.8.*

NOTE: *Documents and fees must be clearly identified as a submission to enter the national state under 35 USC 371 otherwise the submission will be considered as being made under 35 USC 111. 37 C.F.R. § 1.494(f).*

CERTIFICATION UNDER 37 C.F.R. § 1.10*
*(Express Mail label number is **mandatory.**)*
(Express Mail certification is optional.)

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date 9/22/00, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number **EK493794037**, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Deanna M. Rivernider
(type or print name of person mailing paper)

Deanna Rivernider
Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence*

****WARNING:*** *Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg 56,439, at 56,442.*

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. 371:

   a. [X] This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).

   b. [X] The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
[X]*	TOTAL CLAIMS	20- 20 =	0	x $ 18.00 =	$0
	INDEPENDENT CLAIMS	1 - 3 =	0	x $ 78.00 =	$0
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + $260.00				$260.00
BASIC FEE**	[ ] U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: [ ] and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(2) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 CFR 1.492(a)(4)) .... $96.00 [ ] and the above requirements are not met (37 CFR 1.492(a)(1)) .... $670.00 [X] U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the USPTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: [ ] has been paid (37 CFR 1.492(a)(2)) .... $760.00 [ ] has not been paid (37 CFR 1.492(a)(3)) .... $970.00 [X] where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)).......... $840.00				$840.00
	Total of above Calculations				= $1,100.00
SMALL ENTITY	Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed. (note 37 CFR 1.9, 1.27, 1.28)				- $
	Subtotal				$1,100.00
	Total National Fee				$1,100.00
	Fee for recording the enclosed assignment document $40.00 (37 CFR 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				$ 40.00
TOTAL	Total Fees enclosed				$1,140.00

*See attached Preliminary Amendment Reducing the Number of Claims.

   i. [X] A check in the amount of $1,140.00 to cover the above fees is enclosed.

   ii. [ ] Please charge Account No. ___________ in the amount of $ ___________.

   A duplicate copy of this sheet is enclosed.

**WARNING: *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended " 37 C.F.R. § 1 495(b)*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. **[X]** A copy of the International application as filed (35 U.S.C. 371(c)(2)):

NOTE: *Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.*

a. [ ] is transmitted herewith.
b. [ ] is not required, as the application was filed with the United States Receiving Office.
c. **[X]** has been transmitted
  i. **[X]** by the International Bureau.
  Date of mailing of the application (from form PCT/IB/308): 30/09/99 .
  ii. [ ] by applicant on _______.
  Date

4. **[X]** A translation of the International application into the English language (35 U.S.C. 371(c)(2)):
a. **[X]** is transmitted herewith.
b. [ ] is not required as the application was filed in English.
c. [ ] was previously transmitted by applicant on ________________.
Date
d. [ ] will follow.

5. **[X]** Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3)):

NOTE: *The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.*

a. [ ] are transmitted herewith.
b. [ ] have been transmitted
  i. [ ] by the International Bureau.

Date of mailing of the amendment (from form PCT/IB/308): ________.

ii. [ ] by applicant on ______.
Date

c. [X] have not been transmitted as

i. [X] applicant chose not to make amendments under PCT Article 19.
Date of mailing of Search Report (from form PCT/ISA/210): **06/07/99**

ii. [ ] the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. [X] A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. 371(c)(3)):

a. [ ] is transmitted herewith.

b. [ ] is not required as the amendments were made in the English language.

c. [X] has not been transmitted for reasons indicated at point 5(c) above.

7. [X] A copy of the international examination report (PCT/IPEA/409)

[X] is transmitted herewith.

[ ] is not required as the application was filed with the United States Receiving Office.

8. [ ] Annex(es) to the international preliminary examination report

a. [ ] is/are transmitted herewith.

b. [ ] is/are not required as the application was filed with the United States Receiving Office.

9. [ ] A translation of the annexes to the international preliminary examination report

a. [ ] is transmitted herewith.

b. [ ] is not required as the annexes are in the English language.

10. [X] An oath or declaration of the inventor (35 U.S.C. 371(c)(4)) complying with 35 U.S.C. 115

a. [ ] was previously submitted by applicant on __________.
Date

b. [X] is submitted herewith, and such oath or declaration

i. [X] is attached to the application.

ii. [ ] identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. 1.70.

iii. [ ] will follow.

II. Other document(s) or information included:

11. [X] An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):

a. [X] is transmitted herewith.

b. [ ] has been transmitted by the International Bureau.
Date of mailing (from form PCT/IB/308): ______________.

c. [ ] is not required, as the application was searched by the United States International Searching Authority.

d. [ ] will be transmitted promptly upon request.
e. [ ] has been submitted by applicant on ________________.
Date

12. [X] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98:
a. [X] is transmitted herewith.
Also transmitted herewith is/are:
[X] Form PTO-1449 (PTO/SB/08A and 08B).
[X] Copies of citations listed.
b. [ ] will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
c. [ ] was previously submitted by applicant on ________________.
Date

13. [X] An assignment document is transmitted herewith for recording.

A separate [ ] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [X] FORM PTO 1595 is also attached.

14. [X] Additional documents:
a. [X] Copy of request (PCT/RO/101)
b. [X] International Publication No. WO 99/48356
i. [X] Specification, claims and drawing
ii. [ ] Front page only
c. [X] Preliminary amendment (37 C.F.R. § 1.121)
d. [X] Other

Forms PCT/ISA/220, PCT/IB/304, PCT/IB/308, (Written Opinion), PCT/IB/301, PCT/IPEA/416,

15. [X] The above checked items are being transmitted
a. [X] before 30 months from any claimed priority date.
b. [ ] after 30 months.

16. [ ] Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on ____________, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: *"A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).*

09/646825

430 Rec'd PCT/PTO 22 SEP 2000

NOTE. "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

[X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. **04-1105**.

[X] 37 C.F.R. 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

[X] 37 C.F.R. 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

[X] 37 C.F.R. 1.17 (application processing fees)

[X] 37 C.F.R. 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).

[ ] 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . issue fee " From the wording of 37 C.F.R § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

[ ] 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).

SIGNATURE OF PRACTITIONER

Reg. No.: 33,860

Peter F. Corless
(type or print name of practitioner)

EDWARDS & ANGELL, LLP
Dike, Bronstein, Roberts & Cushman, IP Group

Tel. No.: (617) 523-3400

130 Water Street
P.O. Address

Customer No.:

Boston, MA 02109

18/Prts

09/646825
430 Rec'd PCT/PTO 22 SEP 2000

METHOD FOR TRANSFORMING PLANT, THE RESULTANT PLANT AND GENE THEREOF

Technical Field

This invention relates to a method for transforming a useful plant by introducing a gene of another species into the useful plant. More particularly, the present invention pertains the method for transforming the useful plant characterized in that the region of a factor relating to the poly (A) addition of the mRNA of the useful plant to be transformed contained in the base sequence of the gene of the other species is modified into another base sequence not relating to the poly (A) addition of the mRNA without substantially altering the function of the protein encoded by the gene to be introduced, the useful plant produced by it, a nucleic acid in which base sequence used thereto is modified, and a method for producing the said nucleic acid.

Background Arts

Growth of plants needs great numbers of nutrients. The plants absorb most of these nutrients necessary for growth from roots. The plants, which can not absorb nutrients in soil due to having hereditary low enzyme activities required for absorption of nutrients, are known.

For example, iron is an essential element for almost organisms, and is essentially required for large numbers of enzymes involved in functioning cells such as photosynthesis and respiration. Iron solubilized in soil exists mainly in the form of Fe(III) chelate [in some case, Fe(II) chelate]. In general, Fe(II) is prevalently absorbed as compared with Fe(III) by plants, but the absorption depends on plant species.

Plants have two types of mechanisms of iron uptake, i.e. absorption

mechanism (I) (refer to Fig. 1) and absorption mechanism (II) (refer to Fig. 2) (Mori, 1994).

The absorption mechanism (I) shown in Fig. 1 consists of: (1) release of proton into the rhizosphere (Olsen and Brown, 1980), (2) increased reducing activity of Fe(III) in cell membrane of roots (Brown et al., 1961 and Chaney et al., 1972), and (3) excretion of reduced and chelating substances from roots (Hether et al., 1981). Namely, Fe(III) is chelated by the released chelating substance, and Fe(III)-chelate in the free space of roots is reduced to Fe(II) on the cell membrane by ferric-chelate reductase and is absorbed through Fe(II) transporter. It is also thought that the proton is released into the rhizosphere and activity of reductase is increased by lowering pH in the free space. However, the problem is known that since reducing activity of Fe(III) is inhibited by higher pH, strong pH buffering action due to high concentration of carbonate anion results to cause lime chlorosis (Marchner et al., 1986).

Absorption mechanism (II) shown in Fig. 2 is specific to grass and is consisting of: (1) synthesis of mugineic acids (phytosiderophore), (2) release of mugineic acids into the rhizosphere, (3) formation of soluble complex of iron and mugineic acids, and (4) absorption of mugineic acids-iron complex by plants body (Takagi, 1976 and Takagi et al., 1984). The iron uptake mechanism by such the absorption mechanism (II) observed in grass has advantage not to be inhibited by higher pH.

Yeast (Saccharomyces cerevisiae), a model organism of eukaryote, performs iron absorption similar to the above absorption mechanism (I). Since in studies on the gene level in the higher plants, Fe(II) transporter has cloned by complementation of iron absorption mutant of yeast (Eida et al., 1996), no detailed mechanism of iron absorption has been studied.

Contrary to that, the mechanism in yeast (Saccharomyces cerevisiae) has

been studied in detail. Absorption of iron in yeast is initiated by a reduction of Fe(III) to Fe(II) by ferric-chelate reductase FRE1 and FRE2 (Dancis et al., 1990,1992, Georgatsou and Alexandraki, 1994). In the mechanism for uptake of reduced Fe(II) into cells, high affinity absorption mechanism and low affinity absorption mechanism are known.

In the absorption of iron by the high affinity absorption mechanism, after reoxidation of Fe(II) by multicopper oxidase FET3 (Askwith et al., 1994), Fe(III) may be incorporated into cells by ferric transporter FTR1 (Stearman et al., 1996). Copper is required in the reoxidation of divalent iron (Dancis et al., 1994, Klomp et al., 1997), and copper supplying pathway to FET3 has also studied (Yuan et al., 1995 and Lin et al., 1997).

Absorption of iron by the low affinity absorption mechanism may be performed by an action of Fe(II) transporter FET4 (Dix et al., 1994, 1997).

Such the iron absorption mechanism in yeast may be applied to plants, and plants which can be grown in the iron deficient soil may be created.

For that purpose, we have created transgenic tobacco, to which *FRE1* gene of yeast provided by Dr. Dancis (NIH) was transformed (Yamaguchi, 1995).

However, in the transgenic tobacco, to which *FRE1* gene was transformed, the reducing activity was not changed as compared with that of wild type. As a result of Northern hybridization analysis, the transcriptional product of yeast gene *FRE1* in tobacco was so small as 0.9 kb.

Example of such incomplete transcription, in which gene of another species is transformed into the higher plant, is gene group Cry encoding δ -endotoxin (insecticidal protein) of Bacillus thuringiensis. More than 42 Cry genes have been known and are classified into 4 classes (cryI - cryIV)(Whiteley and Schnepf, 1986). The

gene encoding this insecticidal protein was introduced into the higher plant, but neither expression nor extremely low expression was found.

This may be caused by (1) difference in codon usage, (2) high AT content in Cry gene, (3) unstable in mRNA, and (4) a partial splicing of Cry gene as intron.

A preparation of the transgenic plant with high expression of protein has been reported. Namely, in order to express Cry gene group efficiently in the higher plant, base sequence of Cry gene is modified to arrange with base sequence of the plant, and the primer is synthesized, then is completely synthesized by PCR (Perlak et al., 1991, Fujimoto et al., 1993, and Nayak et al., 1997).

Although transformation of the higher plant by introducing gene of the another organism species has known, the expression thereof was not sufficient. Various reasons have been provided as described in the above.

We have made extensive studies on factors for achieving the sufficient expression of a gene in a higher plant which has been transformed by introducing the above gene encoding a protein having a function carried by another organism so as to impart the function in the useful higher plant, and found that base sequence of the factor relating to the poly(A) addition of the mRNA of the transformed plant is an important part of the expression.

Consequently, the present invention provides a method for expressing the introduced gene in the transgenic higher plant with high efficiency, the said transgenic higher plant, and a method for modifying gene therefor.

Disclosure of the Invention

The present invention relates to a method for transforming a useful plant by introducing another gene into the useful plant characterized in that the region of a

factor relating to the poly(A) addition of the mRNA of the useful plant to be transformed contained in the base sequence of the said another gene is modified into another base sequence not relating to the poly(A) addition of the mRNA without substantially altering the function of the protein encoded by the gene to be introduced. Example of the region of a factor relating to the poly(A) addition of the mRNA is preferably AATAAA like base sequence, further the said region of a factor relating to the poly(A) addition of the mRNA is preferably the region existing downstream of the GT-rich base sequence. Further, modification of the base sequence of the said region is preferably carried out based on the codon usage of the transformed useful plant.

In the method of the present invention, it is preferable that base G and T rich region in the gene to be introduced is small; difference in content of base G and C within whole region of the gene to be introduced is small; the sequence has no ATTTA sequence; and/or upstream of the initiation codon of the gene to be transferred has Kozak sequence.

Further, the present invention relates to the transformed useful plant, which can be produced by the method of the present invention. The transformed useful plant of the present invention can be the organism and the seed and has no limitation in the form.

Further, the present invention relates to a nucleic acid, especially DNA, having the modified base sequence, which can be used by the above transforming method.

The base sequence of the nucleic acid of the present invention is a modified base sequence which can be expressed in the transformed useful plant with high efficiency, and, for example, is a factor relating to the poly(A) addition of mRNA of the said useful plant, and is characterized in that a part of factor relating to the said

poly(A) addition is replaced by the other base sequence, further the said base sequence has small G- and T-rich region of the base in the gene to be introduced, has small difference between G- and C-content of the base throughout the gene to be introduced, has no ATTTA sequence and/or preferably the upstream of the initiation codon of the gene to be introduced has Kozak sequence.

Further, the present invention relates to a method for production of the above nucleic acids characterized in that the above nucleic acids are divided into several fragments and these fragments are ligated.

Brief Description of Drawings

Fig. 1: Absorption mechanism (I) of iron in plants.

Fig. 2: Absorption mechanism (II) of iron in plants.

Fig. 3: A position of poly(A) addition in higher plant.

Fig. 4: G- and T-rich sequence in yeast gene *FRE1*.

Fig. 5: Schematic illustration of *refre1* synthesis.

Fig. 6: Sequence of 30 primers used in the synthesis of *refre1*.

Fig. 7: Relationship between *refre1* sequence and primer.

Fig. 8: Preparative scheme of full length *refre1*.

Fig. 9: Total sequence of designed *refre1*.

Fig. 10: Graphical illustration of G- and T-contents in *FRE1* (upper level) and *refre1* calculated by continued 8 base unit.

Fig. 11: Structure of binary vector pRF1.

Fig. 12: A photograph showing growth of transgenic plant of the present invention.

Fig. 13: A photograph showing anthesis of transgenic plant of the present

invention.

Fig. 14: Result of Southern hybridization of the transformant using *refre1* as a probe.

Left: Digestion by *Eco*RI and *Hin*dIII

Right: Digestion by *Hin*dIII

No.1 - No.2: Transformant

W.T: wild type

Fig. 15: Result of Northern hybridization of the transformant using *refre1* as a probe.

No.1 - No.2: Transformant

W.T: wild type.

Fig. 16: A photograph showing activity of ferric-chelate reductase in roots indicating red coloring of BPDS-Fe(II) complex by Fe(II).

Left: Wild type showing no red coloring.

Right: Transformant showing red coloring in roots.

Fig. 17: Photograph showing replicate experiment of the same as in Fig. 16 using another transformant. Red coloring is observed in the transformant (right).

Fig. 18: Photograph showing activity of ferric-chelate reductase by red coloring of BPDS-Fe(II) complex in roots, using second generation of plant obtained from seeds of the transformant. Red coloring of BPDS-Fe(II) complex is observed in the second generation of the transformant (left).

<u>Best Mode for Carrying Out the Invention</u>

The useful plants transformed in the present invention are no limitation, if these are industrially used plants such as foods and pharmaceuticals, and are

preferably higher plants such as grains, vegetables, fruits and tobacco.

Another gene introduced in the present invention is not limited, if it is useful for plants and has no detrimental effects for plants and human. It may be directly useful gene for plants and gene providing resistance against chemicals such as herbicide, and is preferably enzyme derived from organisms such as bacteria and yeast. For example, ferric-chelate reductase FRE1 of yeast involving absorption of iron is preferable.

We have found that in a transformed plant, factors affecting expression of introduced gene may be a base sequence which determines addition of poly(A) of mRNA. Further, we have found that in the upstream of the base sequence, which defines addition of poly(A), GT-rich base sequence is necessary. Namely, in the presence of GT-rich base sequence, addition of poly(A) is determined in plants, subsequently mRNA is split at the position after 10-30 bp from the poly(A) signal, for example AATAAA like base sequence, then poly(A) is added by an action of poly(A) polymerase. Accordingly, in case that the introduced gene has such the base sequence, in the transgenic plants, full length mRNA can not be expressed, and mRNA is split in the position after 10-30 bp from the poly(A) signal having AATAAA like base sequence.

Consequently, the present invention is characterized in that the poly(A) signal of plant in the introduced gene, for example AATAAA like base sequence, preferably GT-rich base sequence, is modified to another base sequence.

A method of design for modifying base sequence is, at first, codon is selected for not to change amino acid sequence encoded by gene to be introduced. Amino acid sequence can be changed, if the sequence has not substantial effect for function for protein, preferably the amino acid sequence may not be changed.

In case that multiple numbers of codons, which encode an amino acid, is

known, the codon having high rate of usage in the plant is preferably selected by considering the codon usage of the plant to be transformed.

Further, not only modification of base sequence of poly(A) signal but also deletion of GT-rich base sequence is preferable. Especially, in case that GT-rich base sequence exists with high proportion, since possibility of splitting mRNA in the region of poly(A) signal like base sequence appearing in the downstream of the GT-rich base sequence is high, a modification for reducing amount of GT content in such the region is important.

Further, in the present invention, in addition to the above modification, it is preferable to modify in order to make smaller difference between G- and C-content of bases throughout the full region of gene to be introduced. More preferably, the sequence should not contain ATTTA sequence, which is known as unstable sequence of mRNA, and/or the sequence has Kozak sequence, which is known as a sequence for effective translation of mRNA in the eukaryote, in the upstream of the initiation codon of gene to be introduced.

The method of the present invention includes a modification of base sequence combined further with usual method of modification to the above modification of base sequence.

The method for modification of base sequence can be made without limitation by known various methods. For example, any conventional method of modification by point mutation and splitting with restriction enzyme can be applied.

Further, in case that large numbers of base have to be modified or gene itself to be introduced has short length, it can be prepared by synthesis. As explained later concretely, even if length of gene is long, the gene is divided into several fragments, and each fragment, which is amplified by PCR, is ligated using restriction enzymes,

then gene having modified base sequence can be prepared.

The method of the present invention is further explained more concretely, but the method of the present invention can not be limited within the scope of the following explanation, and the broad application thereof based on the said explanation can be performed by the person skilled in the art.

We have tried to study the reason why length of mRNA of yeast *FRE1,* which was introduced into tobacco, was short (0.9 kb). As for the reasons for incomplete length of transcriptional product of yeast ferric-chelate reductase *FRE1,* which was introduced in tobacco, two possibilities were considered, i.e.

(1) a part of mRNA was spliced as intron, and

(2) a transcription was terminated within coding region.

As a result of further analysis by RT-PCR, it was found that poly(A) addition occurred within coding region in the transgenic tobacco, to which *FRE1* gene was transformed.

Example of the confirmed expression of yeast gene introduced into the higher plant is invertase (Hincha, 1996). In the present experiment, new knowledge, in which these is a case that full length mRNA can not be synthesized even in the same eukaryotic gene by introducing *FRE1* gene, could be obtained.

Reason why full length mRNA could not be synthesized in the *FRE1* transformed transgenic tobacco was addition of poly(A) within the coding region of *FRE1.*

A poly(A) site is not limited within one position, and in the upstream of each poly(A) site, AAUAAA like base sequence, putative poly(A) signal region was observed. However, although several AAUAAA like sequences were observed at 5'-site of *FRE1,* the poly(A) addition was not observed in these positions.

It may be a GU-rich sequence located in the upstream of the poly(A) signal to determine addition of poly(A) in plant. Namely, if GU-rich sequence exists, addition of poly(A) may be occured in the plant, and in the position of "PyA", which is located at the distance of 10-30 bp from the subsequently appeared AAUAAA like sequence, mRNA is splitted, then the poly(A) may be added by an action of poly(A) polymerase.

In conclusion, the fact that GU-rich sequence, which has no relation to addition of poly(A) in yeast, determines addition of poly(A) in plant, is a cause for not forming full length mRNA in the transgenic tobacco, to which FRE1 is transformed.

A sequence of ferric-chelate reductase *FRE1* having GT-rich region is shown in Fig.4. In Fig. 4, the boxed sequences are thought to be GT-rich regions.

As a result, in order to express ferric-chelate reductase FRE1 in tobacco, deletion of GT-rich sequence from *FRE1* gene may be effective. However, at present, as for the sequence, which determines addition of poly(A) in plant, there may be only known that a consensus sequence may be GU-rich and the sequence is not completely determined. So long as the exact consensus sequence has not be known, there may be possibility not to be obtainable the full length mRNA by only changing the sequence. We have, therefore, tried to design base sequence corresponding to codon usage of plants to be transformed without changing amino acid sequence of FRE1 in order to synthesize full length mRNA in plant.

In order to express yeast ferric-chelate reductase in tobacco, we have redesigned base sequence corresponding well to the codon usage of tobacco without changing amino acid sequence of FRE1. In design of base sequence, the following points are considered.

(1) GT-rich region is eliminated;

(2) Base sequence AATAAA, which may be a poly(A) signal, and the similar base

sequence are eliminated;

(3) In order to confirm easily the base sequence, restriction sites are set at the position in about every 400 bp (417-436 bp), and the sequence is divided in 5 segments;

(4) Base sequence, ATTTA sequence (Ohme-Takagi, 1993), which is called as unstable sequence of mRNA, is eliminated;

(5) In order not to make difference between base content G and C in whole region, position of codons are replaced; and

(6) Kozak sequence, which is a sequence for effectively translating mRNA in eukaryote (Kozak, 1989) is attached prior to the initiation codon.

The thus designed modified base sequence of yeast ferric-chelate reductase FRE1 is shown in Sequence listing, SEQ ID NO: 1. Amino acid sequence thereof is shown in SEQ ID NO: 2.

The designed gene is designated as reconstructed *FRE1* (hereinafter designates as "*refre1*").

The *refre1* of the present invention is synthesized by dividing into 5 segments (A-E) as shown in Fig. 5.

A segment A consists of a sequence of 1-434 bp, in which restriction sites are designed as in base 1: *Eco*RI, base 7: *Xba*I and base 429: *Bam*HI.

A segment B consists of a sequence of 429-845 bp, in which restriction sites are designed as in base 429: *Bam*HI and base 840: *Mro*I.

A segment C consists of a segment of 840-1275 bp, in which restriction sites are designed as in base 840: *Mro*I and base 1270: *Sal*I.

A segment D consists of a segment of 1270-1696 bp, in which restriction sites are designed as in base 1270: *Sal*I and base 1691: *Pst*I.

A segment E consists of a segment of 1691-2092 bp, in which restriction sites

are designed as in base 1691: *Pst*I, base 2081: *Sac*I and base 2087: *Hin*dIII.

Each segment A-E, each consisting of sequence having 417-436 bp, is synthesized using 6 primers having 77-83 mer, respectively. Thirty primers used, from A-1 to E-6, are shown in Fig. 6. These base sequence are shown in sequence listings, SEQ ID NO: 5 - SEQ ID NO: 34.

Among primers in the segments, -1, -2 and -3 are sense strands, and primers -4, -5 and -6 are anti strands. Primers are designed so as to have complementary base sequence consisting of 12 or 13 bp in the 3' end for primers -3 and -4, and overlapping sequence consisting of 12 or 13 bp in 3' end for primers -1 and -2, -2 and -3, -4 and -5, and -5 and -6. The primer -1 and -6 is designed to have restriction site at the base 1 in 5' end.

Relationship between these primers and the designed base sequences is shown in Fig. 7.

Respective segments A-E are prepared by PCR using primers synthesized according to the above base sequence (refer to Fig. 5).

After the reaction mixture of third step PCR was electrophoresed with 0.8% agarose gel, bands having expected length (417 - 436 bp) were cut and purified, then were cloned into plasmid pT7Blue (R) vector (supplied by Takara Corp.). The base sequences of the obtained clones were confirmed and the clones having exact base sequences were selected by applying fluorescent DNA sequencer DSQ-1000L (made by Shimadzu Corp.).

Segments having exact sequences were obtained and full length *refre1* was prepared by applying restriction sites according to methods shown in Fig. 8.

Direction of insertion in segments B and E is essentially required for preparation of full length. In other segments, the segments containing exact base

sequence were used without relation to direction of the insertion.

Total base sequence of the obtained *refre1* is shown in Fig. 9. Specific features of sequence of *refre1* are:

(1) 75.3% of homology to the original *FRE1* (100% homology in amino acid sequence);

(2) To have no sequence consisting of only G or T which is continuously linked more than 8 bases;

(3) It does not contain not only a sequence AATAAA but also sequences replaced by any one of bases in the above sequence;

(4) It does not contain a sequence ATTTA; and

(5) No difference is observed in GC content through the whole region of the sequence.

Fig. 10 shows decreased numbers of sequences consisting of serial G and T in *refre1* as compared with the original *FRE1*. This illustrates GT content of serial 8 bases in the *FRE1* and *refre1* sequences. As shown in Fig. 10, uniformity of GT content in *refre1* is demonstrated as compared with the original *FRE1*.

The thus synthesized gene *refre1* is introduced into tobacco (Nicotiana tabacum L. var. SRI). As a result of transformation, 68 kanamycin resistant plants were reproduced. In order to confirm transformation of the objective gene in the reproduced plant and its copying number, genomic Southern hybridization was conducted. As a result, one to several copied plants of the transformant, *refre1* gene was confirmed.

A method from gene introduce into plant cells to reproduction of plant can be performed by conventional method, for example, as described in "Laboratory Manual on functional analysis of plant gene" (Maruzen) [ref. (4)].

Specifically, a fragment of restriction enzymes, *Xba*I and *Sac*I, in *refre1*, which was cloned with pT7Blue(R) vector by the above method, was exchanged with ORF of

β-glucronidase in the binary vector pBI121 (TOYOBO Co. Ltd.) to prepare binary vector pRF1. The structure of the binary vector pRF1 is shown in Fig. 11.

The thus obtained binary vector pRF1 was transformed into E. coli, and E. coli, which is bearing helper plasmid pRK2013, were shake cultured at 37℃ for overnight. On the other hand, Agrobacterium tumefaciens C58 was shake cultured in LB liquid culture medium 1 ml containing proper antibiotic at 26℃ for 2 nights. Each 100μl thereof was mixed on LB plate without containing antibiotic, cultured at 26℃ for 2 nights, then surface of the plate was scraped by using platinum spatula, and cultured on the selection plate [LB plate containing 100μg/μl rifampicin (Rf) and 25μg/μl kanamycin (Km)] at 26℃ for 2 nights to form single colony.

The single colony was shake cultured in LB (Km and Rf) liquid medium 4 ml at 26℃ for 2 nights. Plasmid was extracted, and restriction enzyme treated cleavage pattern of the plasmid indicated existence of pRF1.

Plant to be transformed was prepared as follows.

Two or three young leaves, size about 8 cm, of wild type tobacco were cut, and were sterilized in a petri dish, filled with sterilized solution (hypochlorous acid 10% and Tween 20, 0.1%), with stirring for 15 minutes. After rinsing three times with sterilized water, the leaves were cut off in 8 mm squares. To the leaves in a petri dish was added the cultured liquid 3 ml of the binary vector bearing Agrobacterium tumefaciens C58, which was cultured 26℃ for 2 nights. After 1 minute, the liquid was rapidly removed by using Pasteur pipette, and residual liquid was removed off on the autoclaved filter paper.

Fragments of leaves were put on a culture medium, added with benzyl adenine and naphthaleneacetic acid to the MS medium, and cultured under light

condition at 25℃ for 3 days. Thereafter, the fragments of leaves were transferred to the medium added with CLAFORAN, and cultured for 1 week further were transferred to the medium added with CLAFORAN and kanamycin, then inoculated in every 2 weeks. When calli were induced and shoots were formed, the shoots were cut off with scalpel and were transferred to the MS medium added with kanamycin.

Shoots with roots were transplanted to the vermiculite and the plants were raised with supplying hyponex (Hyponex Japan Co. Ltd.) to obtain the transgenic plants.

Sixty-eight transgenic plants having kanamycin resistance could be obtained as a result of transformation. Example of photograph of the grown plant is shown in Fig. 12 and the photograph of plant with flower is shown in Fig. 13.

Among them, 5 individual plants were treated with genomic Southern hybridization. Result is shown in Fig. 14.

In the genomic Southern hybridization, extraction of genomic DNA from the transgenic tobacco was performed according to the description in "Plant Cell Technology Series 2, Protocol for PCR Experiments of Plants" (Shujun-Sha) [Ref. (2)]. The obtained genomic DNA was digested by restriction enzymes *Eco*RI and *Hin*dIII and the hybridization was performed by using a probe, which was prepared with full length fragment of *refre1* as a template ([α-32P]-dATP was used).

In the genomic Southern hybridization shown in Fig. 14, amounts of DNA were arranged at the time of restriction enzyme treatment, but deviation was observed due to treating with ethanol after restriction enzyme treatment. Consequently, darkness of bands detected is not always reflecting the copy numbers of the introduced gene.

In digestion with restriction enzymes, *Eco*RI and *Hin*dIII, band, size 3.2 kb,

which was expected in all individuals, was observed. However, in the individual No. 12, a band with slightly smaller than 3.2 kb was detected. According to this result, 1 copy of *refre1* in No. 1 and No. 11, 3 or 4 copies in No. 2 and 4 copies in No. 9 were thought to exist.

In the digestion by *Hin*dIII on No. 12, band could not be detected due to loading failure on the gel.

As a result of the above genomic Southern hybridization analysis, the *refre1* gene was found to be introduced into the selected five individuals.

When the sequence is cleaved by restriction enzymes *Eco*RI and *Hin*dIII, a sequence from promoter to terminator is cleaved, and the introduced *refre1* gene is transcribed to mRNA under regulation of CaMV35S promoter. In the *Eco*RI and *Hin*dIII digestion of No. 12, a reason for detecting a band slightly smaller than 3.2 kb might be due to the fact that one of the introduced construction was cleaved before integration in the plant genom, and was inserted into the position close to *Eco*RI or *Hin*dIII site in the plant genom.

Next, in the transformed tobacco No. 1 and No. 2, in which the introduced *refre1* gene was confirmed by the genomic Southern hybridization hereinbefore, formation of full length mRNA was confirmed by Northern analysis.

In the Northern analysis, a method of blotting was performed, for example, according to the conventional method described in "Cloning and Sequencing" (Noson-Bunka-Sha) [Ref. (1)], and the method in hybridization was performed according to the method as described in Southern analysis hereinbefore.

A result of Northern hybridization is shown in Fig. 15. In Fig. 15, no band is detected in the lane of wild type (W.T.). In lanes of No. 1 and No. 2, major bands with a size of 2.5 kb are detected and several bands smaller than that are detected.

In Northern analysis, formation of the full length mRNA as a result of introducing *refre1* of the present invention could be confirmed. In the present analysis, fundamentally although total RNA should be extracted from the root, in which mRNA is expected to be expressed, in this experiment, if the root is cut, then the plant of the subsequent generation can not be obtained, consequently the extraction has to perform from leaves. Since CaMV35S as a promoter is used and *refre1* gene is expressed in the whole parts of plant, even in the analysis performed by extraction of total RNA from leaves, it is confirmed that the transcription can be performed in leaves and roots of plants and site of addition of poly(A) is not changed.

As a result of Northern hybridization, since the transcriptional product of 2.5 kb was confirmed in the tobacco, to which *refre1* was introduced, poly(A) addition might occur by NOS terminator. Full length mRNA was also found in the tobacco, to which *refre1* was introduced.

A band smaller than the length of 2.5 kb as seen in Fig. 15 is detected at the position corresponding to rRNA as compared with that of photograph after electrophoresis. Although it was thought to be a nonspecific absorption of probe in rRNA, since it was not hybridized with wild type RNA, it is surely hybridized with transcriptional product of *refre1*. There may be possibly produced the shorter mRNA than the full length mRNA in the *refre1*. However, since it is detected as the same length with rRNA, it may be thought that, in the electrophoresis of RNA, mRNA of *refre1* may be dragged by rRNA, which exists in large amount. This reason may be clarified by Northern hybridization with purified poly(A) + RNA, however as obviously shown in Fig. 15, most of mRNA is full length mRNA and there is no reason to trace and clarify such the reason.

In order to perform Northern hybridization, RNA of the transformant of No. 1,

which was found as one copy, and that of the transformants of No. 2, which were found as 3 or 4 copies, were electrophoresed, it was found that the bands of No. 2 were dark colored depending on copy numbers of *refre1* gene.

Further, among the obtained transformed 68 plants of tobacco (selected by kanamycin), constant ferric-chelate reductase in root was confirmed in 6 plants.

For detection of reductase activity, a property of red color formation of the complex of bathophenanthroline disulfonic acid (BPDS), which is a strong chelater for Fe(II), with Fe(II) was applied. After removal of vermiculite from transformant and wild type tobacco, roots were laid on the gel containing BPDS with shield light using aluminum foil and stood at 27℃ for 24 hours. Reduction of Fe(III) was confirmed by coloring in the rhizosphere of the transformant.

Photographs confirming reductase activity are shown in Fig. 16 and Fig. 17. In photographs of Fig. 16 and Fig. 17, red coloring is observed in the transformant of the right photographs as shown with black color.

As shown, ferric-chelate reductase was detected in all of 6 plants (selected by kanamycin), which were used for confirmation of ferric-chelate reductase activity in roots. In order to demonstrate difference between the transformed tobacco and wild type tobacco, reaction time of reductase was set for long time as 24 hours, but the difference was observed at about 1 hour of the reaction time. In all of 6 plants used in the transformation experiments, the leaves, which were put on the gel for detecting activity, showed tendency of crinkle as compared with the condition of wild type leaves. This may be due to involvement in the mechanism of the introduced *refre1* gene expression in the leaves. Though not so many times of activity tests were performed because of this phenomenon, it is clear that *refre1* gene is expressed as a result of transcription and translation in the root under regulation of CaMV35S promoter.

As explained in the above, we have created novel tobacco which could express yeast ferric-chelate reductase FRE1 in the higher plant tobacco.

Reasons for not obtaining full length transcription product of different organism gene may be due to two possibilities including splicing a part of mRNA as an intron, and adding poly(A) within the coding region.

The present invention provides a method for designing base sequence for obtaining full length transcriptional product by transferring gene of different species in the higher plant. In the method of the present invention, in order to avoid addition of poly(A) in the coding region, it was found that it is necessary to design the sequence consisting of continued base sequence of 8 bases or more without containing sequence consisting of only G or T, and to design the sequence without containing not only a sequence of AATAAA but also a sequence, in which any one of bases thereof is replaced by another base (i.e. NATAAA, ANTAAA, AANAAA, AATNAA, AATANA, or AATAAN).

It was also found that to design the sequence, in which G and C contents should be constantly distributed in the full region, is important.

Further, in the concrete explanation of the present invention hereinbefore, since CaMV35S was used as a promoter, ferric-chelate reductase was expressed in the transformed tobacco of the whole plant. As shown, locally expressing gene can be expressed in the systemic plant as a result of combining with the promoter. On the contrary, the expressing gene in the systemic plant can be expressed in the local region by using combination with preferable promoter.

A mechanism of absorption by reduction of Fe(III) is specific to iron acquiring mechanism in the monocots and dicots except for grass, and also the grass may absorb iron by reducing Fe(III) to Fe(II) under the condition of sufficient iron. As a result of ligating the ferric-chelate reductase gene *refre1* of the present invention with a

promoter, which is specifically active in the root under iron deficient condition, novel grass, in which iron absorption mechanism (I) and absorption mechanism (II) under the condition of iron deficiency can be functioned, may able to be created.

References in the present invention are listed hereinbelow.

(1) Cloning and Sequence (1989), Noson-Bunka-Sha

(2) Protocol of PCR Experiments for Plants (1995), Shujun-Sha

(3) Biological Experiments, Illustrated Fundamentals of Genetic Analysis (1995), Shujun-Sha

(4) Labolatory Manual, Functional Analysis of Plant Genes (1992), Maruzen

(5) Askwith, C., et al., Cell 76: 403-410 (1994)

(6) Brown, J.C., et al., Soil Sci. 91: 127-132 (1961)

(7) Chaney, R.L., et al., Plant Physiol. 50: 208-213 (1972)

(8) Dancis, A., et al., Mol. Cell. Biol. 10: 2293-2301 (1990)

(9) Dancis, A., et al., Proc. Natl. Acad. Sci. USA 89: 3869-3873 (1992)

(10) Dix, D.R., et al., J. Biol. Chem. 269: 26092-26099 (1994)

(11) Dix, D., et al., J. Biol. Chem. 272: 11770-11777

(12) Eide, D., et al., Proc. Natl. Acad. Sci. USA 93: 5624-5628 (1996)

(13) Fujimoto, H., et al., Biol/Technology 11: 1151-1155 (1993)

(14) Gallie, D.R., et al., Plant Cell 9: 667-673 (1997)

(15) Georgatsou, E., et al., Mol. Cell. Biol. 14: 3065-3073

(16) Guo, Z., et al., Biochem. Sci. 21: 477-481 (1996)

(17) Hassett, R., et al., J. Biol. Chem. 270: 128-134 (1995)

(18) Hether, N.H., et al., J. Plant Nutr. 7: 667-676 (1984)

(19) Hincha, D.K., et al., J. Plant Physiol. 147: 604-610 (1996)

(20) Kozak, M., J. Cell Biol. 108: 229-241 (1989)

(21) Lin, S.J., et al., J. Biol. Chem. 272: 9215-9220 (1997)

(22) Marschner, H., et al., J. Plant Nutr. 9: 695-713 (1986)

(23) Mewes, H.W., et al., Nature 387: 7-8 (1997)

(24) Mori, S., (1994), Biochemistry of metal micronutrients in the rizosphere. (Eds. Monthey, J.A., Crowley, D.E., Luster, D.G., Lewis Publishers), pp225-249

(25) Naito, S., et al., Plant Mol. Biol. 11: 109-124 (1988)

(26) Nakanishi, H., et al., Plant Cell Physiol. 34: 401-410 (1933)

(27) Nayak, P., et al., Proc. Natl. Acad. Sci. USA 94: 2111-2116 (1997)

(28) Ohme –Takagi, M., et al., Proc. Natl. Acad. Sci. USA 90: 11811-11815 (1993)

(29) Okumura, N., et al., J. Plant Nutr. 15: 2157-2172 (1992)

(30) Okumura, N., et al., Plant Mol. Biol. 25: 705-719 (1994)

(31) Olsen, R.A., et al., J. Plant Nutr. 2: 629-660 (1980)

(32) Perlak, F.J., et al., Proc. Natl. Acad. Sci. USA 88: 3324-3328 (1991)

(33) Stearman, R., et al., Science 271: 1552-1557 (1996)

(34) Takagi, S., Soil Sci. Plant Nutr. 22: 423-433 (1976)

(35) Takagi, S., et al., J. Plant Nutr. 7: 469-477 (1984)

(36) Whiteley, H.R., et al., Annu. Rev. Microbiol. 40: 549-576 (1986)

(37) Wu, L., et al., Plant J. 8: 323-329 (1995)

(38) Yuan, D.S., et al., Proc. Natl. Acad. Sci. USA 92: 2632-2636 (1995)

Examples

The present invention will be explained in detail hereinbelow in examples, but is not construed as limiting within these examples.

In the examples hereinbelow, fundamental gene manipulation is performed

according to the description of "Cloning and Sequencing" (Noson-Bunka-Sha) and analysis of base sequence of gene is performed by using DNASIS (made by Hitachi Corp.).

Example 1 (Extraction of total RNA from *FRE1* introduced transgenic tobacco)

Extraction of total RNA from *FRE1* introduced transgenic tobacco was performed according to a method described in the reference (Naito et al., 1988).

Leaves 2g of *FRE1* introduced transgenic tobacco were put in the mortar, and liquid nitrogen was added thereto, then leaves were completely mashed. Three fold amounts of buffer for extraction and equal amount of phenol/chloroform (1 : 1) were added to the debris and suspended, then centrifuged at 8000 rpm for 15 minutes, and extracted with chloroform once. Ethanol precipitation was conducted at -80℃ for 30 minutes, and centrifuged at 4℃ for 30 minutes. Precipitate was washed with 70% ethanol and dried in vacuum. The precipitate was dissolved in DEPC treated water 1 ml, centrifuged at 13500 rpm for 3 minutes, and the supernatant was transferred to a new tube, further 10 M LiCl, 1/4 volume, was added and allowed to stand on ice for 2 hours. The mixture was centrifuged at 12000 rpm at 4℃ for 10 minutes, then the precipitate was washed with 70% ethanol and dried in vacuo. The dried product was dissolved in DEPC treated water 50μl.

Reagent buffer for extraction

1M Tris HCl pH 9.0

1% SDS

(β-mercaptoethanol 120μl was added to 6 ml of buffer before use)

Example 2 (Purification of poly(A) + RNA and synthesis of cDNA)

Poly(A) + RNA was purified from total RNA 100μg obtained in example 1 by applying with Dynabeads Oligo (dT) 25 (DYNAL Inc.). This poly(A) + RNA was treated with reverse transcription reaction by M-MLV reverse transcriptase (TOYOBO Co. Ltd.) at 37℃ for 1 hour using the following hybrid primer to obtain cDNA.

Hybrid primer (dT^{17} adapter primer):

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTT-3'

Example 3 (RT-PCR and confirmation of base sequence)

PCR was conducted with the primer specific to hybrid primer and the 5' primer of *FRE1* using cDNA obtained in example 2 as a template.

Reaction product of PCR was electrophoresed with 0.8% agarose gel, and the obtained band was cloned to pT7Blue(R) vector (Takara Corp.). Colony was shake cultured in LB medium for overnight, extracted the plasmid by alkaline-SDS method, and the base sequence of 7 clones, to which the insertion was confirmed by restriction enzyme treatment, was determined by using Bca BEST DNA polymerase ("Biotechnology Experiments Illustrated, Fundamentals of gene analysis") (Shujun-Sha).

Primer specific to hybrid primer:

5'-GACTCGAGTCGACATCG-3'

5' primer of FRE1:

5'-ACACTTATTAGCACTTCATGTATT-3'

Reaction condition for PCR:

(1) 95℃ for 5 minutes;

(2) 95℃ for 40 seconds;

(3) 55℃ for 30 seconds;

(4) 72℃ for 1 minute;

(5) 72℃ for 10 minutes; and

(6) 4℃

In the above procedures, (2), (3) and (4) were repeated 40 times.

As a result, in the transgenic tobacco transformed with *FRE1*, poly(A) was attached at the position as shown in Fig. 3, in the transcribed mRNA from *FRE1* gene.

Attached points of poly(A) were not uniform, and several length of mRNA was observed. A sequence, which might be recognized as poly(A) signal at the upstream of poly(A) site, was indicated as putative poly(A) signal.

<u>Example 4 (Production of each segment by PCR)</u>

Each segment was prepared by PCR as illustrated in Fig. 5. The super Taq (Sawady Inc.) was used as Taq polymerase.

Composition of PCR reaction is as follows.

PCR reaction solution in the first step:

10 x buffer	10 μl
2 mM dNTP mixture	10 μl
20 μM primer (-3)	5 μl
20 μM primer (-4)	5 μl

distilled water to total volume 99.5 μl

PCR reaction solution in the second step:

PCR reaction solution in the first step	1 μl
10 x buffer	10 μl

2 mM dNTP mixture	10 μl
20μM primer (-2)	5 μl
20μM primer (-5)	5 μl
distilled water to total volume	99.5 μl

PCR reaction solution in the third step:

PCR reaction solution in the second step	1μl
10 x buffer	10μl
2 mM dNTP mixture	10 μl
20μM primer (-1)	5 μl
20μM primer (-6)	5 μl
distilled water to total volume	99.5 μl

Reaction conditions for PCR:

(1) 95℃ for 5 minutes;

(2) add Taq 0.5 μl

(3) 95℃ for 40 seconds;

(4) 45℃ for 1 minute;

(5) 72℃ for 1 minute;

(6) 94℃ for 40 seconds;

(7) 60℃ for 30 seconds;

(8) 72℃ for 1 minute;

(9) 72℃ for 10 minutes;

(10) 4℃

The above procedures of (3), (4) and (5) were repeated 5 times, and the procedures of (6), (7) and (8)) were repeated 20 times, respectively.

Example 5 (Cloning and confirmation of base sequence)

After electrophoresis of PCR reaction solution in the third step in example 4 with 0.8% agarose gel, a band, which had expected length (417 - 436 bp), was cleaved and purified, then was cloned into the plasmid pT7Blue (R) vector (Takara Inc.). The base sequence of the thus obtained clone was confirmed and the exact base sequence was selected using SHIMADZU luminescent DNA sequencer DSQ-1000L.

After obtaining segment of each exact sequence, full length of *refre1* was prepared as shown in Fig. 8 by applying with restriction enzyme sites. A direction of insertion of the segment B and E was essential for preparing the full length. As for the other segments, the sequence containing exact base sequence was used without relation to the direction of insertion.

Full length of base sequence of the synthesized *refre1* is shown in sequence listing SEQ ID NO: 1 and Fig. 9.

Example 6 (Introduction of *refre1* into tobacco)

A gene *refre1* synthesized in example 5 was introduced into tobacco (*Nicotiana tabacum* L. var. SRI). As a result of transformation, 68 individual plants resistant to kanamycin were generated. Genomic Southern hybridization was performed in order to confirm introduction of *refre1*, an objective gene, in the generated plant and copying number thereof. As a result, existence of one to several copies of *refre1* gene was confirmed.

A method from gene introduce into plant cells to generation of plant was performed according to description in "Laboratory Manual for Functional Analysis of Plant Genes" (Maruzen).

(1) Preparation of binary vector pRF1 for transformation

*Xba*I and *Sac*I fragments of ***refre1***, which were cloned in pT7Blue (R) vector, were exchanged with ORF of β-glucronidase of binary vector pBI121 to prepare a binary vector pRF1. A structure of the binary vector pRF1 is shown in Fig. 11.

(2) Transfer of binary vector pRF1 into Agrobacterium

Agrobacterium tumefaciens C58 was shake cultured at 26℃ for 2 nights in LB liquid medium 1ml containing suitable antibiotic, and E. coli having pRF1 and E. coli having helper plasmid pRK2013 were shake cultured at 37℃ for one night in LB liquid medium 1ml containing suitable antibiotic. Each 100 μl was mixed on the LB plate without containing antibiotics. After the mixture was cultured at 26℃ for 2 nights, plate surface was scraped out the plate using platinum loop and incubated to form single colony on the selection plate [LB plate containing 100 μg/μl rifampicin (Rf) and 25 μg/μl kanamycin (Km)] (at 26℃ for 2 nights).

The thus obtained single colony was shake cultured in LB (Km and Rf) liquid medium 4 ml at 26℃ for 2 nights, and the plasmid was extracted by alkaline-SDS method, then existence of pRF1 was confirmed by observing cleavage patterns by restriction enzymes.

(3) Infection of Agrobacterium to tobacco and regeneration of plant

Two or three young leaves of tobacco (Nicotiana tabacum L. var. SRI), size about 8 cm, were cut, put them into the petri dish filled with sterilized water (hypochlorous acid 10% and Tween 20, 0.1%), and sterilized with stirring for 15 minutes. The leaves were rinsed with sterilized water for 3 times, and were cut in 8

mm square using scalpel. Cultured liquid of Agrobacterium 3 ml having binary vector pRF1 cultured at 26℃ for 2 nights was added to fragments of leaves in the petri dish.

After one minute, the liquid was immediately removed off by using Pasture pipette and the residual liquid was removed off using autoclaved sterilized filter paper. The leaves were put on the MS medium (II) hereinbelow and cultured at 25℃ for 3 days under lighting condition. Thereafter fragments of leaves were transferred to MS medium (III) and cultured for 1 week, then transferred to the MS medium (IV) and subcultured in every 2 weeks. When calli were induced and shoots were formed, the shoots were cut using scalpel and transferred to the MS medium (V). The shoots with roots were inoculated to vermiculite, and raised with supplying hyponex (Hyponex Japan Co., Ltd) to obtain the regenerated plant.

The compositions of MS medium for tobacco used in the experiments hereinbefore are as follows.

Major elements (g/l)

NH_4NO_3	1.65
KNO_3	1.9
$CaCl_2 \cdot 2H_2O$	0.44
$MgSO_4 \cdot 7H_2O$	0.44
KH_2PO_4	0.17

Minor elements (mg/l)

H_3BO_4	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83

$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Fe(III)Na-EDTA	0.042 mg/l
myo-inositol	100 mg/l
thiamine	5 mg/l
sucrose	30 g/l
geranylated g	2 g/l

MS medium (I) was prepared by the composition hereinbefore. The other MS media were prepared by adding the following phytohormone and/or antibiotics to the MS medium (I).

Phytohormone	benzyladenine (BA)	1.0 mg/l
	naphthaleneacetic acid (NAA)	0.1 mg/l
Antibiotics	kanamycin	100 mg/l
	claforan	200 mg/l

MS medium (II) MS medium (I) + BA + NAA

MS medium (III) MS medium (I) + BA + NAA + claforan

MS medium (IV) MS medium (I) + BA + NAA + claforan + kanamycin

MS medium (V) MS medium (I) + kanamycin

Example 7 (Southern analysis)

(1) Extraction of genomic DNA from tobacco

Extraction of genomic DNA from tobacco was performed according to the method described in "Plant Cell Engineering Series: Protocol for PCR Experiments in

Plants" (Shujun-Sha).

Leaves 0.1 - 0.2g were put in the mortar, and liquid nitrogen was added thereto, then leaves were completely mashed. The crushed leaves were put into the Eppendorf tube, and 2% CTAB solution 300 μl was added and mixed, then treated at 65℃ for 30 minutes. Equal amount of chloroform and isoamyl alcohol (24 : 1) was added and mixed for 5 minutes.

The mixture was centrifuged at 12000 rpm for 15 minutes, and the upper layer was transferred to the new tube, then chloroform-isoamyl alcohol extraction was repeated once again, and the upper layer was transferred to the new tube. 1 - 1.5 volume of 1% CTAB solution was added, mixed, allowed standing at room temperature for 1 hour, and centrifuged at 8000 rpm for 10 minutes. The upper layer was discarded and 1M CsCl 400 μl was added to the residue, and heated at 65℃ until complete dissolving the precipitate. 100% ethanol 800μl was added thereto, mixed, allowed to standing at -20℃ for 20 minutes, then centrifuged at 12000 rpm for 5 minutes. The upper layer was discarded, and the residue was washed with 70% ethanol, dried in vacuum and dissolved in TE buffer 30μl.

Reagents	2% CTAB solution	
	Tris-HCl (pH 8.0)	100 mM
	EDTA (pH 8.0)	20 mM
	NaCl	1.4 M
	CTAB (cetyltrimethylammonium bromide)	2 %
	1% CTAB solution	
	Tris-HCl (pH 8.0)	50 mM
	EDTA (pH 8.0)	20 mM

CTAB	1 %

(2) Cleavage of genomic DNA by restriction enzyme and electrophoresis

Restriction enzyme treatments were performed by digestion using *Eco*RI and *Hin*dIII, by which sequence from pCaMV35S to tNOS was cleaved, and by digestion using only *Hin*dIII, by which sequence of upstream of pCaMV35S was cleaved.

Genomic DNA 10 μg with the reaction volume 100 μl was treated by restriction enzyme for overnight, precipitated by adding ethanol and dissolved the precipitate in TE buffer 20μl. To the solution was added the loading buffer 2μl, and the solution was electrophoresed with 0.8% agarose gel at 60V for 5 hours. After completion of electrophoresis, gel was stained with ethidium bromide and photographed on the UV transluminater with the scale.

(3) Blotting and hybridization

Gel after photographing was washed with distilled water, and was shaken in 0.2 N HCl for 10 minutes. A method of blotting was performed according to the description in "Cloning and Sequencing" (Noson-Bunka-Sha). The gel was transferred to nylon membrane (New Hybond-N+ Amersham) with 0.4 N NaOH, and the membrane washed with 2 x SSPE for 5 minutes, and dried at room temperature for 3 hours. A method of hybridization was referred with "Biotechnology Experiments Illustrated, Fundamentals of Gene Analysis" (Shujun-Sha) The membrane was treated for prehybridization with prehybridization buffer 30 ml, which was previously warmed at 65℃, for 1 hour at 65℃, and the hybridization buffer was exchanged (25 ml). Probe was added and hybridization was performed at 65℃ for 12 hours. The membrane was washed with washing solution, which was previously warmed at 65℃,

twice at 65℃ for 10 minutes, and was washed once with high stringent washing solution at 65℃ for 10 minutes. The membrane was wrapped with Saran wrap, exposed on imaging plate for 24 hours, and result was confirmed by image analyzer (Fuji Photo Film Co. Ltd.).

Reagents

20 x SSPE

NaCl	3 M
NaH_2PO_4	0.2 M
EDTA	1 mM

1M Church phosphate buffer

$NaHPO_4$ 0.5 mol was added to distilled water about 800 ml, adjusted pH to 7.2 by H_3PO_4, then filled up to 1 liter by adding distilled water, and autoclaved.

Hybridization buffer

Church phosphate buffer	0.5 M
EDTA	1 mM
SDS (v/v)	7 %

Denatured salmon sperm (1 mg/ml) 1/100 vol. was added before use.

Washing solution

Church phosphate buffer	40 mM
SDS (v/v)	1 %

High stringent washing solution

0.2 x SSPE	
SDS (v/v)	0.1 %

(4) Preparation of probe

Probe was prepared by random primer DNA labeling kit ver. 2.0 (Takara Corp.) using full length *refre1* as a template (proviso that [α-^{32}P]-dATP was used), and non-reacted [α-^{32}P]-dATP was removed using Probe Quant TM G-50 Micro Columns (Pharmacia, Biotech Inc.).

Result is shown in Fig. 14. The left side in Fig. 14 shows digestion using restriction enzymes *Eco*RI and *Hin*dIII, and the right side in Fig. 14 shows digestion using only *Hin*dIII. In Fig. 14, W.T. means wild type.

Example 8 (Northern analysis)

(1) Extraction of total RNA

Total RNA was extracted from leaves of the transgenic tobacco, to which *refre1* gene was introduced, and leaves of the wilt type tobacco according to the same method as described in example 1.

(2) Electrophoresis of RNA

Electrophoresis vessel, gel receiver, comb and Erlenmeyer flask were treated previously with abSolve (RNase inhibitor, Du Pont Inc.). 20 x MOPS 10 ml, agarose 2.4 g and sterilized distilled water 100 ml were poured into Erlenmeyer flask, and agarose was dissolved using microwave oven. Formaldehyde 10 ml was added to the gel which was cooled to about 50°C, and sterilized distilled water was added up to 200 ml, which was used when gelification occurred. 1 x MOPS about 800 ml was added in the electrophoresis vessel, and added ethidium bromide 10 mg/ml thereto for use as electrophoresis buffer. RNA sample buffer 16 μl was added to the total RNA 10 μg, filled up to 20 μl with sterilized distilled water, and the mixture was warmed at 65°C

for 10 minutes, then allowed to standing for 5 minutes on ice, and was electrophoresed. Electrophoresis condition was that after electrophoresis was performed at 60 V for 1 hour, further electrophoresis was performed at 120 V for 2 hours.

Reagents:

20 x MOPS

MOPS	0.4 M
NaOAc	0.1 M
EDTA	0.02 M

RNA sample buffer

Formaldehyde	1.6 ml
Formamide	5.0 ml
20 x MOPS	0.5 ml
glycerol pigment solution	1.6 ml
Total	8.7 ml

Glycerol pigment solution

glycerol	5 ml
bromophenol blue	1 mg
xylenecyanol	1 mg
0.5 M EDTA (pH 8.0)	0.02 ml

(3) Blotting and hybridization

After electrophoresis, gel was set on UV illuminator and photographed with the scale. A method of blotting was followed according to the description in "Cloning

and Sequencing" (Noson-Bunka-Sha). Namely, RNA was transferred from gel to nylon membrane (New Hybond-N, Amersham Inc.) with 20 x SSPE. After 12 hours, the membrane was washed with 2 x SSPE for 5 minutes, and dried at room temperature for 3 hours, then RNA was fixed on the membrane by irradiating with UV for 5 minutes.

A method of hybridization was performed as same as the case of Southern analysis.

Result is shown in Fig. 15. In Fig. 15, W.T. indicates wild type. No band was detected in the lane of wild type (W.T.). In No. 1 and No. 2 lanes, major band was detected at the size of 2.5 kb, and several bands were detected in the lower position thereof.

<u>Example 9 (Confirmation of ferric-chelate reductase)</u>

The transgenic tobacco, to which *refre1* gene was introduced, and wild type tobacco were transplanted in vermiculite and raised with supplying hyponex. Ferric-chelate reductase activity was confirmed by using plants, about 5 cm - 10 cm.

For confirmation of ferric-chelate reductase activity, red coloring generated by formation of complex with bathophenanthroline disulfonic acid (BPDS), which was strong chelating agent for Fe(II), and Fe(II) was applied. Agarose was added to assay buffer up to 0.4%, dissolved by using microwave oven, and cooled. 500 μM Fe(III)-EDTA, 1/100 vol., and 500 μM BPDS, 1/100 vol., were added to the slightly cooled gel, and stirred to put in the vessel, then waited for solidification. After removed off vermiculite from the transformant and wild type tobacco, roots were laid on the gel, and shielded from light and allowed to standing at 27°C for 24 hours.

The similar experiment was performed using second generation of the

transgenic plant, which was germinated from seeds of the regenerated plant. Reaction time in this experiment was set for 1 hour.

Assay buffer

$CaSO_4$	0.2 mM
MES buffer pH 5.5	5.0 mM

Photographs showing confirmation of ferric-chelate reductase activity are shown in Fig. 16 and Fig. 17. Photograph showing confirmation of ferric-chelate reductase activity of the second generation plant is shown in Fig. 18. Reduction of Fe(III) was confirmed as a result of coloring of the transformant in the rhizosphere.

Claims

1. A method for transforming a useful plant by introducing a gene of another species into the useful plant wherein the region of a factor relating to the poly (A) addition of the mRNA of the useful plant to be transformed contained in the base sequence of the gene of the other species is modified into another base sequence not relating to the poly (A) addition of the mRNA without substantially altering the function of the protein encoded by the gene to be introduced.

2. The method according to claim 1, wherein the gene of another species to be introduced is derived from yeast.

3. The method according to claim 1 or 2, wherein the region of a factor relating to the poly (A) addition of the mRNA is a base sequence having AATAAA like sequence.

4. The method according to claim 3, wherein the region of a factor relating to the poly (A) addition of the mRNA is located in a downstream from the GT-rich base sequence.

5. The method according to any one of claims 1-4, wherein the modification of base sequence in the region of a factor relating to the poly (A) addition of the mRNA is performed based on a codon usage of the useful higher plant to be transformed.

6. The method according to any one of claims 1-5, wherein the modification of base sequence is performed so that the region rich in base G and base T is reduced.

7. The method according to any one of claims 1-6, wherein the modification of base sequence comprises small difference between base G and base C covering throughout the region of gene to be introduced.

8. The method according to any one of claims 1-7, wherein the modification of base sequence is performed so as not to have ATTTA sequence.

9. The method according to any one of claims 1-8, characterized by having Kozak sequence in the upstream of the initiation codon of the gene to be introduced.

10. The method according to any one of claims 1-9, wherein the gene to be introduced encodes a protein involved in absorption of nutrition.

11. The method according to claim 10, wherein the gene to be introduced is the gene encoding ferric-chelate reductase FRE1.

12. The method according to claim 11, wherein the gene encoding ferric-chelate reductase FRE1 is derived from yeast.

13. The method according to any one of claims 1-12, wherein the useful plant is grass.

14. The method according to any one of claims 1-12, wherein the useful plant is tobacco.

15. A transformed useful plant which can be produced by the method according to claims 1-14.

16. The plant according to claim 15, wherein the plant is seed.

17. A nucleic acid having modified base sequence which can be used by the method according to any one of claims 1-14.

18. The nucleic acid according to claim17, wherein the nucleic acid is DNA.

19. The DNA according to claim 18, wherein the gene to be introduced is the DNA encoding ferric-chelate reductase FRE1.

20. The DNA according to claim 19, wherein the DNA has a base sequence of SEQ ID NO:1.

21. A method for producing the nucleic acid according to any one of claims 17-20, wherein the nucleic acid is cleaved into several fragments and these fragments are ligated.

ABSTRACT

A method for achieving the sufficient expression of a gene in a useful higher plant which has been transformed by transferring the above gene encoding a protein having a function carried by another organism so as to impart the function to the plant. Namely, a method for transforming a useful plant by transferring a gene of another species into the plant characterized in that the region of a factor relating to the poly(A) addition of the mRNA of the useful plant to be transformed contained in the base sequence of the gene of the other species is denatured into another base sequence not relating to the poly(A) addition of the mRNA without substantially altering the function of the protein encoded by the gene to be transferred; and a gene usable in the gene transfer.

Fig. 1

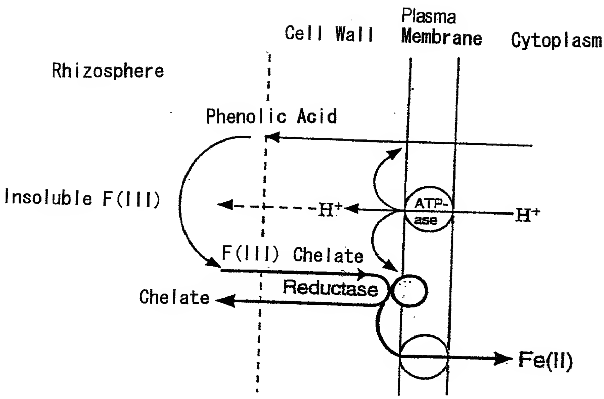
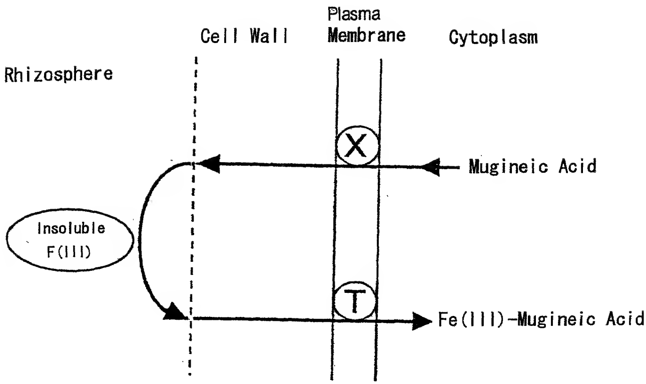

Plasma Membrane
Cell Wall
Cytoplasm
Rhizosphere
Phenolic Acid
Insoluble F(III)
ATP-ase
H+
H+
F(III) Chelate
Reductase
Chelate
Fe(II)

Fig. 2



Two Kinds of Fe-Uptake Mechanisms in Higher Plants

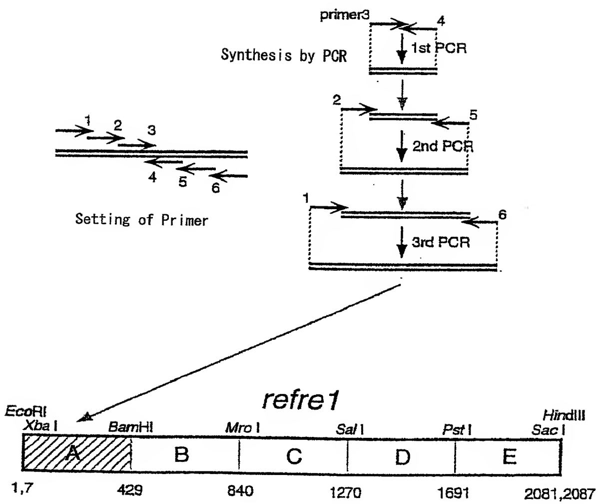
Fig. 3

```
        putative         poly(A)                                putative            poly(A)
    poly(A) signal        site                              poly(A) signal           site
541 TCCGTCAAAAAATCACTTATTTATCCTTCTGTTTACAAAGATTATAATGAACGAACTTTTTATTTATGGAAGCGTCTACCATTTAATTTT 630
181 S  V  K  K  S  L  I  Y  P  S  V  Y  K  D  Y  N  E  R  T  F  Y  L  W  K  R  L  P  F  N  F 210

                             putative       poly(A)    poly(A)
                         poly(A) signal      site       site
631 ACAACTCGAGGCAAGGGTCTCGTCGTATTAATTTTTGTTATTTTGACTATATTATCTCTCAGTTTTGGTCATAATATTAAACTTCCACAC 720
211 T  T  R  G  K  G  L  V  V  L  I  F  V  I  L  T  I  L  S  L  S  F  G  H  N  I  K  L  P  H 240
```

Fig. 4

```
   1 ATGGTTAGAACCCGTGTATTATTCTGCTTATTTATATCTTTTTTTGCTACGGTTCAATCG   60
  61 AGTGCTACACTTATTAGCACTTCATGTATTTCCCAAGCTGCGCTATACCAATTTGGATGT  120
 121 TCTAGTAAATCTAAAAGTTGCTACTGTAAAAACATCAATTGGCTGGGTTCAGTGACAGCA  180
 181 TGTGCCTATGAGAATTCCAAATCTAACAAAACACTAGACAGCGCCTTAATGAAGTTAGCA  240
 241 TCCCAATGTTCAAGCATCAAAGTTTATACTTTAGAGGACATGAAGAATATTTATTTAAAT  300
 301 GCGTCAAATTATTTGAGAGCACCTGAGAAAAGTGATAAAAAAACCGTGGTTAGTCAAGCG  360
 361 CTCATGGCGAACGAGACAGCGTATCATTATTATTATGAGGAAAATTATGGTATCCATCTT  420
 421 AACCTAATGCGCTCTCAATGGTGCGGTTGGGGTCGGTCTTCTTGTGGGTGGCTGTGGTTT  480
 481 ACTGCAGCCACTATCTTGAACATTCTGAAAAGGGTGTTTGGTAAGAACATCATGGCAAAC  540
 541 TCCGTCAAAAAATCACTTATTTATCCTTCTGTTTACAAAGATTATAATGAACGAACTTTT  600
 601 TATTTATGGAAGCGTCTACCATTTAATTTTACAACTCGAGGCAAGGGTCTCGTCGTATTA  660
 661 ATTTTTGTTATTTTGACTATATTATCTCTCAGTTTTGGTCATAATATTAAACTTCCACAC  720
 721 CCATATGATAGGCCCAGATGGAGAAGAAGTATGGCCTTTGTGAGTCGTAGAGCAGACTTG  780
 781 ATGGCCATTGCACTTTTTCCCAGTAGTCTATCTATTCGGAATAAGAAATAATCCCTTCATC  840
 841 CCTATAACAGGGCTTTCCTTTTCTACATTTAATTTCTATCATAAATGGTCTGCCTACGTT  900
 901 TGTTTCATGTTGGCCGTTGTACACTCAATTGTCATGACCGCCTCGGGAGTGAAAAGAGGT  960
 961 GTGTTTCAAAGTCTGGTTAGGAAATTTTACTTTAGGTGGGGTATAGTGGCAACGATATTA 1020
1021 ATGTCTATTATTATTTTCCAAAGTGAAAAAGTATTTAGAAATAGAGGGTATGAGATATTC 1080
1081 CTTCTTATTCATAAAGCGATGAATATTATGTTCATTATTGCCATGTACTACCATTGTCAC 1140
1141 ACCCTGGGGTGGATGGGTTGGATTTGGTCAATGGCTGGTATTTTATGCTTTGATAGATTC 1200
1201 TGCAGGATTGTTAGAATAATCATGAATGGTGGCTTGAAAACTGCTACTTTGAGTACCACT 1260
1261 GATGATTCTAATGTTATTAAAATTTCAGTAAAAAAACCAAAGTTTTTCAAGTACCAAGTA 1320
1321 GGAGCTTTCGCATACATGTATTTCTTATCACCAAAAAGTGCATGGTTCTATAGTTTCCAA 1380
1381 TCACATCCATTTACAGTATTATCGGAACGACACCGTGATCCAAACAATCCAGATCAATTG 1440
1441 ACGATGTACGTAAAGGCAAATAAAGGTATCACTCGAGTTTTGTTATCGAAAGTTCTAAGT 1500
1501 GCTCCAAATCATACTGTTGATTGTAAAATATTCCTTGAAGGCCCATATGGTGTAACGGTT 1560
1561 CCACATATCGCTAAGCTAAAAAGAAATCTGGTAGGTGTAGCCGCTGGTTTGGGTGGTGCG 1620
1621 GCTATTTATCCGCACTTTGTCGAATGTTTACGGTTACCATCTACTGATCAACTTCAGCAT 1680
1681 AAATTTTACTGGATTGTTAATGACCTATCCCATTTGAAATGGTTTGAAAATGAATTGCAA 1740
1741 TGGTTAAAGGAGAAAAGTTGTGAAGTCTCAGTCATATATACTGGTTCCAGTGTTGAGGAC 1800
1801 ACAAATTCAGATGAGAGTACAAAAGGTTTTGATGATAAAGAAGAAAGCGAAATCACTGTT 1860
1861 GAATGTCTCAATAAAAGACCTGATTTGAAAGAACTAGTGCGCTCGGAAATAAAACTCTCA 1920
1921 GAACTAGAGAATAATAATATTACCTTTTATTCCTGCGGGCCAGCAACGTTTAACGACGAT 1980
1981 TTTAGAAATGCAGTGGTCCAAGGTATAGACTCTTCCTTGAAGATTGACGTTGAACTAGAA 2040
2041 GAAGAAAGTTTTACATGGT                                         2059
```

Fig. 5



primer3
4
1st PCR
Synthesis by PCR
2
5
2nd PCR
1
6
3rd PCR
1
2
3
4
5
6
Setting of Primer
refre1
EcoRI
XbaI
BamHI
MroI
SalI
PstI
HindIII
SacI
A
B
C
D
E
1,7
429
840
1270
1691
2081,2087

Fig. 6

Sequence Name	Base Sequence (5' → 3')	
A-1	GAATTCTCTAGACTCCACCATGGTTAGAACCAGAGTCCTTTTCTGCCTCTTCATCTCTTTCTTCGCTACAGTCCAATCGAGCG	83mer
A-2	GTCCAATCGAGCGCTACACTCATCTCCACTTCATGCATTTCTCAGGCTGCACTGTACCAGTTCGGATGCTCAAGCAAGTCAAA	83mer
A-3	CAAGCAAGTCAAAGTCTTGCTACTGCAAGAACATCAATTGGCTCGGAAGCGTCACTGCATGCGCTTATGAGAACTCCAAATCT	83mer
A-4	TCCAGTGTGTAAACCTTGATACTTGAGCATTGGCTGGCAAGTTTCATCAAAGCGGAGTCCAGAGTCTTGTTAGATTTGGAGTT	83mer
A-5	TGTCTTCTTATCGGATTTCTCAGGAGCGCGAAGGTAGTTACTTGCATTAAGGTAGATGTTCTTCATGTCCTCCAGTGTGTAAA	83mer
A-6	GGATCCCATAGTTTTCCTCATAGTAGTAGTGATAGGCCGTCTCATTTGCCATCAACGGTTGTGAAACAACTGTCTTCTTATCG	83mer
B-1	GGATCCACTTGAATTTGATGCGATCTCAATGGTGCGCATGGGGCCTCGTCTTCTTCTGGGTCGCAGTCCTTACCGCCGCA	80mer
B-2	CCTTACCGCCGCAACTATCTTGAACATTCTCAAACGCGTATTCGGCAAGAACATTATGGCAAATTCTGTTAAGAAGTCTC	80mer
B-3	GTTAAGAAGTCTCTTATCTACCCAAGCGTTTACAAAGACTACAACGAGAGAACTTTCTATCTTTGGAAACGTTTGCCATT	80mer
B-4	AGAGTGAGAGAATAGTCAGAATGACAAAGATAAGAACTACGAGTCCTTTGCCTCGAGTTGTAAAGTTGAATGGCAAACGT	80mer
B-5	AATGCCATTGATCTTCTCCATCTAGGTCTATCGTAAGGATGTGGCAACTTGATGTTATGTCCGAAAGAGAGTGAGAGAAT	80mer
B-6	TCCGGATACCGAAAAGGTACACCACGGGGAAAAGAGCGATTGCCATCAAGTCAGCACGGCGTGAGACGAATGCCATTGAT	80mer
C-1	TCCGGAACAACCCCTTCATCCCAATCACCGGATTGAGCTTTAGTACTTTCAACTTTTACCACAAATGGTCAGCATACGTCTGC	83mer
C-2	GCATACGTCTGCTTCATGTTAGCCGTCGTCCATTCAATCGTTATGACCGCTTCAGGAGTTAAACGAGGAGTATTCCAGTCTCT	83mer
C-3	TATTCCAGTCTCTTGTAAGGAAATTCTACTTCAGATGGGGAATAGTAGCCACAATTCTTATGTCCATCATCATTTTCCAGTCC	83mer
C-4	ATAAACATGATGTTCATGGCTTTGTGAATAAGTAAGAAGATTTCATAACCTCGGTTCCTGAAGACCTTCTCGGACTGGAAAAT	83mer
C-5	GAGGATGCCAGCCATGGACCAGATCCAGCCCATCCATCCTAGTGTGTGGCAATGGTAATACATAGCTATGATAAACATGATGT	83mer
C-6	GTCGACAAAGTGGCGGTCTTAAGACCTCCGTTCATGATGATACGTACAATTCGGCAGAACCTGTCGAAGCAGAGGATGCCAGC	83mer
D-1	GTCGACCACAGATGATTCTAACGTTATCAAGATCTCTGTCAAGAAGCCTAAGTTCTTCAAGTATCAAGTGGAGCATTTGCC	82mer
D-2	GGAGCATTTGCCTATATGTACTTTCTTTCACCAAAATCAGCCTGGTTCTACAGTTTTCAATCTCATCCCTTCACAGTCCTAT	82mer
D-3	TTCACAGTCCTATCAGAAAGGCACAGAGATCCTAACAACCCAGATCAACTAACTATGTACGTCAAAGCTAACAAGGGCATTA	82mer
D-4	CCTCTAAGAAAATCTTGCAATCAACGGTATGGTTTGGAGCGCTTAGAACTTTGCTAAGAAGTACTCTCGTAATGCCCTTGTT	82mer
D-5	GGCCCGCAGCTACTCCTACTAGATTTCTCTTAAGTTTGGCAATGTGAGGGACAGTTACGCCATATGGTCCCTCTAAGAAAAT	82mer
D-6	CTGCAGTTGATCAGTGCTAGGCAATCTAAGGCATTCTACGAAATGGGGGTAGATGGCTGCCACGCCGAGGCCCGCAGCTACT	82mer
E-1	CTGCAGCACAAGTTCTACTGGATCGTCAACGACCTTAGTCACCTTAAGTGGTTCGAAAACGAGCTACAATGGCTTAA	77mer
E-2	ACAATGGCTTAAGGAGAAATCTTGTGAAGTCTCTGTCATCTACACTGGGTCATCAGTGGAGGATACAAACTCAGATG	77mer
E-3	CAAACTCAGATGAGTCCACTAAGGGTTTCGATGACAAGGAAGAATCTGAAATCACCGTAGAATGCCTTAACAAGAGG	77mer
E-4	GTGATGTTGTTGTTCTCGAGTTCTGACAATTTGATCTCTGATCTCACTAGCTCTTTGAGGTCTGGCCTCTTGTTAAG	77mer
E-5	CGATACCTTGTACAACTGCATTCCTAAAGTCGTCATTGAAAGTCGCTGGTCCGCATGAGTAGAAAGTGATGTTGTTG	77mer
E-6	AAGCTTGAGCTCTTACCAAGTAAAACTCTCCTCCTCTAGTTCGACATCTATCTTCAGACTAGAATCGATACCTTGTA	77mer

Fig. 7

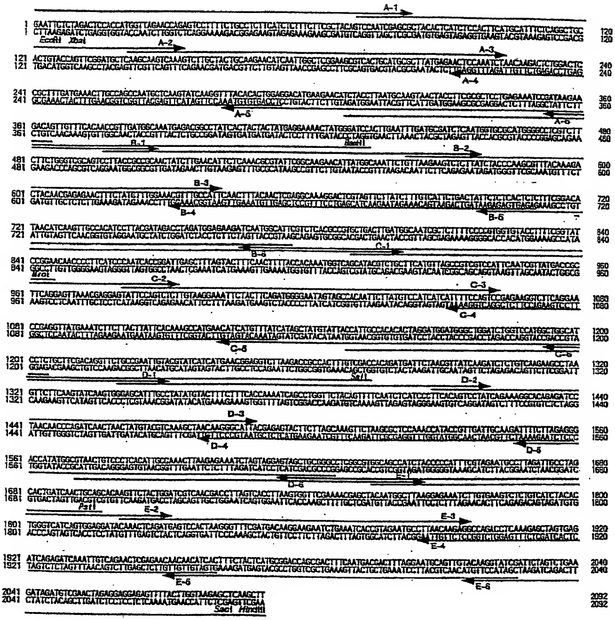

A-1
A-2
A-3
A-4
A-5
A-6
B-1
B-2
B-3
B-4
B-5
B-6
C-1
C-2
C-3
C-4
C-5
C-6
D-1
D-2
D-3
D-4
D-5
D-6
E-1
E-2
E-3
E-4
E-5
E-6
EcoRI
XbaI
BamHI
MroI
SalI
PstI
SacI
HindIII

Fig. 8

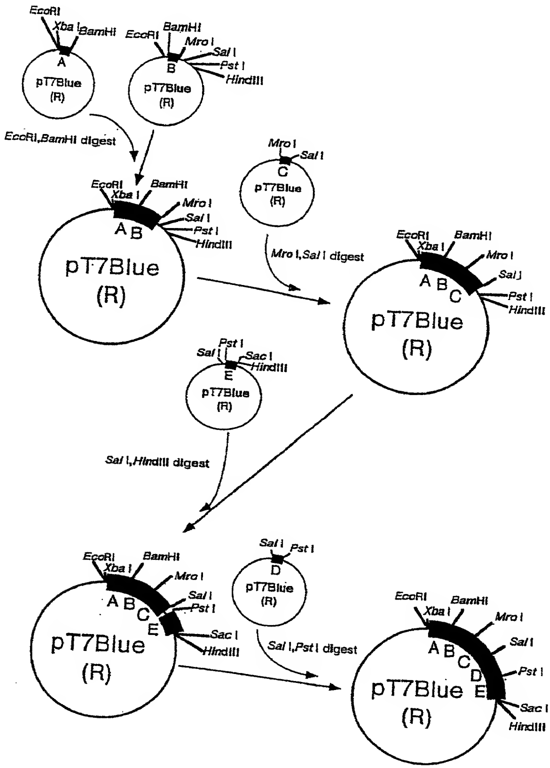
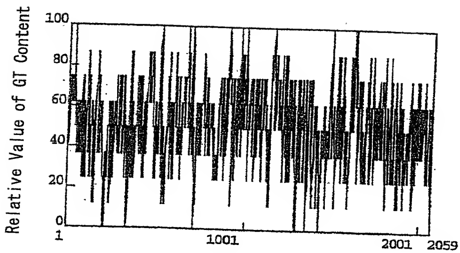

EcoRI
Xba I
BamHI
A
pT7Blue
(R)
EcoRI
BamHI
Mro I
Sal I
Pst I
HindIII
B
pT7Blue
(R)
EcoRI,BamHI digest
Mro I
Sal I
C
pT7Blue
(R)
EcoRI
Xba I
BamHI
Mro I
Sal I
Pst I
HindIII
A B
pT7Blue
(R)
Mro I,Sal I digest
EcoRI
Xba I
BamHI
Mro I
Sal I
Pst I
HindIII
A B C
pT7Blue
(R)
Pst I
Sal I
Sac I
HindIII
E
pT7Blue
(R)
Sal I,HindIII digest
Sal I
Pst I
D
pT7Blue
(R)
EcoRI
Xba I
BamHI
Mro I
Sal I
Pst I
A B C E
Sac I
HindIII
pT7Blue
(R)
Sal I,Pst I digest
EcoRI
Xba I
BamHI
Mro I
Sal I
Pst I
A B C D E
Sac I
HindIII
pT7Blue
(R)

Fig. 9

```
1                                                                     gaattctctagactccacc   19

20   ATGGTTAGAACCAGAGTCCTTTTTTGCCTCTTCATCTCTTTCTTCGCTACAGTCCAATCGAGCGCTACACTCATCTCCACTTCATGCATT   109
1    M  V  R  T  R  V  L  F  C  L  F  I  S  F  F  A  T  V  Q  S  S  A  T  L  I  S  T  S  C  I    30

110  TCTCAGGCTGCACTGTACCAGTTCGGATGCTCAAGCAAGTCAAAGTCTTGCTACTGCAAGAACATCAATTGGCTCGGAAGCGTCACTGCA   199
31   S  Q  A  A  L  Y  Q  F  G  C  S  S  K  S  K  S  C  Y  C  K  N  I  N  W  L  G  S  V  T  A    60

200  TGCGCTTATGAGAACTCCAAATCTAACAAGACTCTGGACTCCGCTTTGATGAAACTTGCCAGCCAATGCTCAAGTATCAAGGTTTACACA   289
61   C  A  Y  E  N  S  K  S  N  K  T  L  D  S  A  L  M  K  L  A  S  Q  C  S  S  I  K  V  Y  T    90

290  CTGGAGGACATGAAGAACATCTACCTTAATGCAAGTAACTACCTTCGCGCTCCTGAGAAATCCGATAAGAAGACAGTTGTTTCACAACCG   379
91   L  E  D  M  K  N  I  Y  L  N  A  S  N  Y  L  R  A  P  E  K  S  D  K  K  T  V  V  S  Q  P    120

380  TTGATGGCAAATGAGACGGCCTATCACTACTACTATGAGGAAAACTATGGGATCCACTTGAATTTGATGCGATCTCAATGGTGCGCATGG   469
121  L  M  A  N  E  T  A  Y  H  Y  Y  Y  E  E  N  Y  G  I  H  L  N  L  M  R  S  Q  W  C  A  W    150

470  GGCCTCGTCTTCTTCTGGGTCGCAGTCCTTACCGCCGCAACTATCTTGAACATTCTCAAACGCGTATTCGGCAAGAACATTATGGCAAAT   559
151  G  L  V  F  F  W  V  A  V  L  T  A  A  T  I  L  N  I  L  K  R  V  F  G  K  N  I  M  A  N    180

560  TCTGTTAAGAAGTCTCTTATCTACCCAAGCGTTTACAAAGACTACAACGAGAGAACTTTCTATCTTTGGAAACGTTTGCCATTCAACTTT   649
181  S  V  K  K  S  L  I  Y  P  S  V  Y  K  D  Y  N  E  R  T  F  Y  L  W  K  R  L  P  F  N  F    210

650  ACAACTCGAGGCAAAGGACTCGTAGTTCTTATCTTTGTCATTCTGACTATTCTCTCACTCTCTTTCGGACATAACATCAAGTTGCCACAT   739
211  T  T  R  G  K  G  L  V  V  L  I  F  V  I  L  T  I  L  S  L  S  F  G  H  N  I  K  L  P  H    240

740  CCTTACGATAGACCTAGATGGAGAAGATCAATGGCATTCGTCTCACGCCGTGCTGACTTGATGGCAATCGCTCTTTTCCCCGTGGTGTAC   829
241  P  Y  D  R  P  R  W  R  R  S  M  A  F  V  S  R  R  A  D  L  M  A  I  A  L  F  P  V  V  Y    270

830  CTTTTCGGTATCCGGAACAACCCCTTCATCCCAATCACCGGATTGAGCTTTAGTACTTTCAACTTTTACCACAAATGGTCAGCATACGTC   919
271  L  F  G  I  R  N  N  P  F  I  P  I  T  G  L  S  F  S  T  F  N  F  Y  H  K  W  S  A  Y  V    300

920  TGCTTCATGTTAGCCGTCGTCCATTCAATCGTTATGACCGCTTCAGGAGTTAAACGAGGAGTATTCCAGTCTCTTGTAAGGAAATTCTAC   1009
301  C  F  M  L  A  V  V  H  S  I  V  M  T  A  S  G  V  K  R  G  V  F  Q  S  L  V  R  K  F  Y    330

1010 TTCAGATGGGGAATAGTAGCCACAATTCTTATGTCCATCATCATTTTCCAGTCCGAGAAGGTCTTCAGGAACCGAGGTTATGAAATCTTC   1099
331  F  R  W  G  I  V  A  T  I  L  M  S  I  I  I  F  Q  S  E  K  V  F  R  N  R  G  Y  E  I  F    360

1100 TTACTTATTCACAAAGCCATGAACATCATGTTTATCATAGCTATGTATTACCATTGCCACACACTAGGATGGATGGGCTGGATCTGGTCC   1189
361  L  L  I  H  K  A  M  N  I  M  F  I  I  A  M  Y  Y  H  C  H  T  L  G  W  M  G  W  I  W  S    390

1190 ATGGCTGGCATCCTCTGCTTCGACAGGTTCTGCCGAATTGTACGTATCATCATGAACGGAGGTCTTAAGACCGCCACTTTGTCGACCACA   1279
391  M  A  G  I  L  C  F  D  R  F  C  R  I  V  R  I  I  M  N  G  G  L  K  T  A  T  L  S  T  T    420

1280 GATGATTCTAACGTTATCAAGATCTCTGTCAAGAAGCCTAAGTTCTTCAAGTATCAAGTGGGAGCATTTGCCTATATGTACTTTCTTTCA   1369
421  D  D  S  N  V  I  K  I  S  V  K  K  P  K  F  F  K  Y  Q  V  G  A  F  A  Y  M  Y  F  L  S    450

1370 CCAAAATCAGCCTGGTTCTACAGTTTTCAATCTCATCCCTTCACAGTCCTATCAGAAAGGCACAGAGATCCTAACAACCCAGATCAACTA   1459
451  P  K  S  A  W  F  Y  S  F  Q  S  H  P  F  T  V  L  S  E  R  H  R  D  P  N  N  P  D  Q  L    480

1460 ACTATGTACGTCAAAGCTAACAAGGGCATTACGAGAGTACTTCTTAGCAAAGTTCTAAGCGCTCCAAACCATACCGTTGATTGCAAGATT   1549
481  T  M  Y  V  K  A  N  K  G  I  T  R  V  L  L  S  K  V  L  S  A  P  N  H  T  V  D  C  K  I    510

1550 TTCTTAGAGGGACCATATGGCGTAACTGTCCCTCACATTGCCAAACTTAAGAGAAATCTAGTAGGAGTAGCTGCGGGCCTCGGCGTGGCA   1639
511  F  L  E  G  P  Y  G  V  T  V  P  H  I  A  K  L  K  R  N  L  V  G  V  A  A  G  L  G  V  A    540

1640 GCCATCTACCCCCATTTCGTAGAATGCCTTAGATTGCCTAGCACTGATCAACTGCAGCACAAGTTCTACTGGATCGTCAACGACCTTAGT   1729
541  A  I  Y  P  H  F  V  E  C  L  R  L  P  S  T  D  Q  L  Q  H  K  F  Y  W  I  V  N  D  L  S    570

1730 CACCTTAAGTGGTTCGAAAACGAGCTACAATGGCTTAAGGAGAAATCTTGTGAAGTCTCTGTCATCTACACTGGGTCATCAGTGGAGGAT   1819
571  H  L  K  W  F  E  N  E  L  Q  W  L  K  E  K  S  C  E  V  S  V  I  Y  T  G  S  S  V  E  D    600

1820 ACAAACTCAGATGAGTCCACTAAGGGTTTCGATGACAAGGAAGAATCTGAAATCACCGTAGAATGCCTTAACAAGAGGCCAGACCTCAAA   1909
601  T  N  S  D  E  S  T  K  G  F  D  D  K  E  E  S  E  I  T  V  E  C  L  N  K  R  P  D  L  K    630

1910 GAGCTAGTGAGATCAGAGATCAAATTGTCAGAACTCGAGAACAACAACATCACTTTCTACTCATGCGGACCAGCGACTTTCAATGACGAC   1999
631  E  L  V  R  S  E  I  K  L  S  E  L  E  N  N  N  I  T  F  Y  S  C  G  P  A  T  F  N  D  D    660

2000 TTTAGGAATGCAGTTGTACAAGGTATCGATTCTAGTCTGAAGATAGATGTCGAACTAGAGGAGGAGAGTTTTACTTGGTAAgagctcaag   2089
651  F  R  N  A  V  V  Q  G  I  D  S  S  L  K  I  D  V  E  L  E  E  E  S  F  T  W  *             687

2090 ctt                                                                                          2092
```

Fig. 10

FRE1



refre1

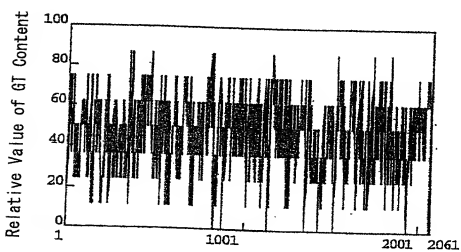


Fig. 11

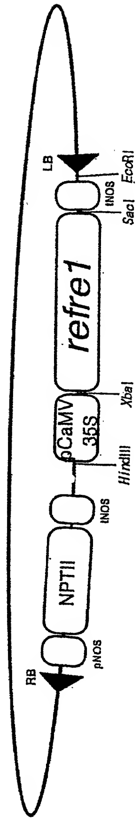
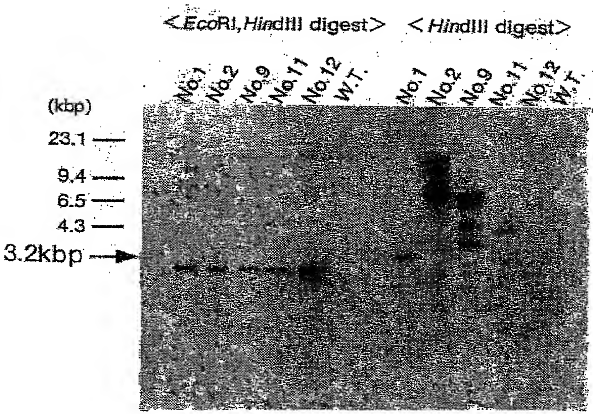

LB
EcoRI
tNOS
SacI
refre1
XbaI
pCaMV
35S
HindIII
tNOS
NPTII
pNOS
RB

Fig. 12

Fig. 13

Fig. 14



<EcoRI, HindIII digest>
<HindIII digest>
No.1
No.2
No.9
No.11
No.12
W.T.
No.1
No.2
No.9
No.11
No.12
W.T.
(kbp)
23.1
9.4
6.5
4.3
3.2kbp

Fig. 15

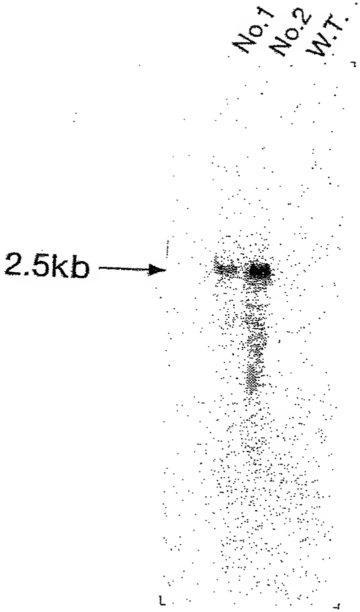


Fig. 16

Fig. 17

Fig. 18

T_2 Plants

Declaration and Power of Attorney for Patent Application
English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR TRANSFORMING PLANT, THE RESULTANT PLANT AND GENE THEREOF

the specification of which

(check one)

[**X**] corresponds to PCT/JP99/01481, filed March 24, 1999
[ ] was filed on ______________________ as United States Application No. or PCT Application No. ______________________
and was amended on ______________________
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
10-96637/1998	Japan	March 24, 1998	[ ]
(Number)	(Country)	(Day/Month/Year Filed)	
______	______	______	[ ]
(Number)	(Country)	(Day/Month/Year Filed)	
______	______	______	[ ]
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. Section 120 of the United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark office all information known to me to be material to patentability as defined in Title 37, C.F.C., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
PCT/JP99/01481	March 24, 1999	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

14

David G. Conlin	Reg. No. 27,026	Christine C. O'Day	Reg. No. 38,256
George W. Neuner	Reg. No. 26,964	Robert L. Buchanan	Reg. No. 40,927
Linda M. Buckley	Reg. No. 31,003	David E. Tucker	Reg. No. 27,840
Peter J. Manus	Reg. No. 26,766	Lisa Swiszcz Hazzard	Reg. No. 44,368
Peter F. Corless	Reg. No. 33,860	George W. Hartnell	Reg. No. 42,639
Cara Z. Lowen	Reg. No. 38,227	Jennifer K. Holmes	Reg. No. 46,778
William J. Daley, Jr.	Reg. No. 35,487	Kerri Pollard Schray	Reg. No. 47,066

Send Correspondence to:

Peter F. Corless
EDWARDS & ANGELL, LLP
Dike, Bronstein, Roberts & Cushman, IP Group
130 Water Street
Boston, Massachusetts 02109
USA

Direct Telephone Calls to:
(name and telephone number)

Peter F. Corless
Telephone: (617) 523-3400
Facsimile: (617) 523-6440

1-00

Full name of sole or first inventor	
Satoshi MORI	
Sole or first inventor's signature: Satoshi Mori	Date: 22-Aug-2000
Residence: 6-7-2-301, Yatsu, Narashino-shi, Chiba-ken, 275-0026 JAPAN JPX	
Citizenship: Japan	
Post Office Address: Same As Above	

2-00

Full name of second inventor	
Hiromi NAKANISHI	
Second inventor's signature: Hiromi Nakanishi	Date: 29 Aug 2000
Residence: 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032 JAPAN JPX	
Citizenship: Japan	
Post Office Address: Same As Above	

3-00

Full name of third inventor Hiroyuki OKI	
Third inventor's signature Hiroyuki Oki	Date: 25 Aug, 2000
Residence 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032 JAPAN JPX	
Citizenship Japan	
Post Office Address Same As Above	

4-00

Full name of sole or fourth inventor Hirotaka YAMAGUCHI	
Fourth inventor's signature Hirotaka Yamaguchi	Date: 23 - Aug - 2000
Residence 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-0032 JAPAN JPX	
Citizenship Japan	
Post Office Address Same As Above	

09/646825

430 Rec'd PCT/PTO 22 SEP 2000

SEQUENCE LISTING

Applicant name : Japan Science And Technology Corporation

Title of invention : Method for transforming plant, the resultant plant and gene thereof

File reference : JA908155

Application : International application filed on March 24, 1999

Filing date : March 24, 1999

Priolity application : JP Patent application No. 10-96637

Priolity application filing date: March 24, 1998

Number of SEQ ID Nos : 34

SEQ ID No.: 1

Length : 2092

Type : nucleic acid

Strandness: double

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : modified base

SEQ :

```
GAATTCTCTA GACTCCACCA TGGTTAGAAC CAGAGTCCTT TTCTGCCTCT TCATCTCTTT   60

CTTCGCTACA GTCCAATCGA GCGCTACACT CATCTCCACT TCATGCATTT CTCAGGCTGC  120

ACTGTACCAG TTCGGATGCT CAAGCAAGTC AAAGTCTTGC TACTGCAAGA ACATCAATTG  180
```

GCTCGGAAGC GTCACTGCAT GCGCTTATGA GAACTCCAAA TCTAACAAGA CTCTGGACTC 240

CGCTTTGATG AAACTTGCCA GCCAATGCTC AAGTATCAAG GTTTACACAC TGGAGGACAT 300

GAAGAACATC TACCTTAATG CAAGTAACTA CCTTCGCGCT CCTGAGAAAT CCGATAAGAA 360

GACAGTTGTT TCACAACCGT TGATGGCAAA TGAGACGGCC TATCACTACT ACTATGAGGA 420

AAACTATGGG ATCCACTTGA ATTTGATGCG ATCTCAATGG TGCGCATGGG GCCTCGTCTT 480

CTTCTGGGTC GCAGTCCTTA CCGCCGCAAC TATCTTGAAC ATTCTCAAAC GCGTATTCGG 540

CAAGAACATT ATGGCAAATT CTGTTAAGAA GTCTCTTATC TACCCAAGCG TTTACAAAGA 600

CTACAACGAG AGAACTTTCT ATCTTTGGAA ACGTTTGCCA TTCAACTTTA CAACTCGAGG 660

CAAAGGACTC GTAGTTCTTA TCTTTGTCAT TCTGACTATT CTCTCACTCT CTTTCGGACA 720

TAACATCAAG TTGCCACATC CTTACGATAG ACCTAGATGG AGAAGATCAA TGGCATTCGT 780

CTCACGCCGT GCTGACTTGA TGGCAATCGC TCTTTTCCCC GTGGTGTACC TTTTCGGTAT 840

CCGGAACAAC CCCTTCATCC CAATCACCGG ATTGAGCTTT AGTACTTTCA ACTTTTACCA 900

CAAATGGTCA GCATACGTCT GCTTCATGTT AGCCGTCGTC CATTCAATCG TTATGACCGC 960

TTCAGGAGTT AAACGAGGAG TATTCCAGTC TCTTGTAAGG AAATTCTACT TCAGATGGGG 1020

AATAGTAGCC ACAATTCTTA TGTCCATCAT CATTTTCCAG TCCGAGAAGG TCTTCAGGAA 1080

CCGAGGTTAT GAAATCTTCT TACTTATTCA CAAAGCCATG AACATCATGT TTATCATAGC 1140

TATGTATTAC CATTGCCACA CACTAGGATG GATGGGCTGG ATCTGGTCCA TGGCTGGCAT 1200

CCTCTGCTTC GACAGGTTCT GCCGAATTGT ACGTATCATC ATGAACGGAG GTCTTAAGAC 1260

CGCCACTTTG TCGACCACAG ATGATTCTAA CGTTATCAAG ATCTCTGTCA AGAAGCCTAA 1320

GTTCTTCAAG TATCAAGTGG GAGCATTTGC CTATATGTAC TTTCTTTCAC CAAAATCAGC 1380

CTGGTTCTAC AGTTTTCAAT CTCATCCCTT CACAGTCCTA TCAGAAAGGC ACAGAGATCC 1440

TAACAACCCA GATCAACTAA CTATGTACGT CAAAGCTAAC AAGGGCATTA CGAGAGTACT 1500

TCTTAGCAAA GTTCTAAGCG CTCCAAACCA TACCGTTGAT TGCAAGATTT TCTTAGAGGG 1560

ACCATATGGC GTAACTGTCC CTCACATTGC CAAACTTAAG AGAAATCTAG TAGGAGTAGC 1620

TGCGGGCCTC GGCGTGGCAG CCATCTACCC CCATTTCGTA GAATGCCTTA GATTGCCTAG 1680

CACTGATCAA CTGCAGCACA AGTTCTACTG GATCGTCAAC GACCTTAGTC ACCTTAAGTG 1740

GTTCGAAAAC GAGCTACAAT GGCTTAAGGA GAAATCTTGT GAAGTCTCTG TCATCTACAC 1800

TGGGTCATCA GTGGAGGATA CAAACTCAGA TGAGTCCACT AAGGGTTTCG ATGACAAGGA 1860

AGAATCTGAA ATCACCGTAG AATGCCTTAA CAAGAGGCCA GACCTCAAAG AGCTAGTGAG 1920

ATCAGAGATC AAATTGTCAG AACTCGAGAA CAACAACATC ACTTTCTACT CATGCGGACC 1980

AGCGACTTTC AATGACGACT TTAGGAATGC AGTTGTACAA GGTATCGATT CTAGTCTGAA 2040

GATAGATGTC GAACTAGAGG AGGAGAGTTT TACTTGGTAA GAGCTCAAGC TT 2092

SEQ ID No.: 2

Length : 687

Type : amino acids

Topology : linear

Molecular type: protein

Original Source

Organism : yeast

SEQ :

Met Val Arg Thr Arg Val Leu Phe Cys Leu Phe Ile Ser Phe Phe 15

Ala Thr Val Gln Ser Ser Ala Thr Leu Ile Ser Thr Ser Cys Ile 30

Ser Gln Ala Ala Leu Tyr Gln Phe Gly Cys Ser Ser Lys Ser Lys 45

Ser Cys Tyr Cys Lys Asn Ile Asn Trp Leu Gly Ser Val Thr Ala 60

Cys Ala Tyr Glu Asn Ser Lys Ser Asn Lys Thr Leu Asp Ser Ala 75

Leu Met Lys Leu Ala Ser Gln Cys Ser Ser Ile Lys Val Tyr Thr 90

Leu Glu Asp Met Lys Asn Ile Tyr Leu Asn Ala Ser Asn Tyr Leu 105

Arg Ala Pro Glu Lys Ser Asp Lys Lys Thr Val Val Ser Gln Pro 120

Leu Met Ala Asn Glu Thr Ala Tyr His Tyr Tyr Tyr Glu Glu Asn 135

Tyr Gly Ile His Leu Asn Leu Met Arg Ser Gln Trp Cys Ala Trp 150

Gly Leu Val Phe Phe Trp Val Ala Val Leu Thr Ala Ala Thr Ile 165

Leu Asn Ile Leu Lys Arg Val Phe Gly Lys Asn Ile Met Ala Asn 180

Ser Val Lys Lys Ser Leu Ile Tyr Pro Ser Val Tyr Lys Asp Tyr 195

Asn Glu Arg Thr Phe Tyr Leu Trp Lys Arg Leu Pro Phe Asn Phe 210

Thr Thr Arg Gly Lys Gly Leu Val Val Leu Ile Phe Val Ile Leu 225

Thr Ile Leu Ser Leu Ser Phe Gly His Asn Ile Lys Leu Pro His 240

Pro Tyr Asp Arg Pro Arg Trp Arg Arg Ser Met Ala Phe Val Ser 255

Arg Arg Ala Asp Leu Met Ala Ile Ala Leu Phe Pro Val Val Tyr 270

Leu Phe Gly Ile Arg Asn Asn Pro Phe Ile Pro Ile Thr Gly Leu 285

Ser Phe Ser Thr Phe Asn Phe Tyr His Lys Trp Ser Ala Tyr Val 300

Cys Phe Met Leu Ala Val Val His Ser Ile Val Met Thr Ala Ser 315

Gly Val Lys Arg Gly Val Phe Gln Ser Leu Val Arg Lys Phe Tyr 330

Phe Arg Trp Gly Ile Val Ala Thr Ile Leu Met Ser Ile Ile Ile 345

Phe Gln Ser Glu Lys Val Phe Arg Asn Arg Gly Tyr Glu Ile Phe 360

Leu Leu Ile His Lys Ala Met Asn Ile Met Phe Ile Ile Ala Met 375

Tyr Tyr His Cys His Thr Leu Gly Trp Met Gly Trp Ile Trp Ser 390

Met Ala Gly Ile Leu Cys Phe Asp Arg Phe Cys Arg Ile Val Arg 405

Ile Ile Met Asn Gly Gly Leu Lys Thr Ala Thr Leu Ser Thr Thr 420

Asp Asp Ser Asn Val Ile Lys Ile Ser Val Lys Lys Pro Lys Phe 435

Phe Lys Tyr Gln Val Gly Ala Phe Ala Tyr Met Tyr Phe Leu Ser 450

Pro Lys Ser Ala Trp Phe Tyr Ser Phe Gln Ser His Pro Phe Thr 465

Val Leu Ser Glu Arg His Arg Asp Pro Asn Asn Pro Asp Gln Leu 480

Thr Met Tyr Val Lys Ala Asn Lys Gly Ile Thr Arg Val Leu Leu 495

Ser Lys Val Leu Ser Ala Pro Asn His Thr Val Asp Cys Lys Ile 510

Phe Leu Glu Gly Pro Tyr Gly Val Thr Val Pro His Ile Ala Lys 525

Leu Lys Arg Asn Leu Val Gly Val Ala Ala Gly Leu Gly Val Ala 540

Ala Ile Tyr Pro His Phe Val Glu Cys Leu Arg Leu Pro Ser Thr 555

Asp Gln Leu Gln His Lys Phe Tyr Trp Ile Val Asn Asp Leu Ser 570

His Leu Lys Trp Phe Glu Asn Glu Leu Gln Trp Leu Lys Glu Lys 585

Ser Cys Glu Val Ser Val Ile Tyr Thr Gly Ser Ser Val Glu Asp 600

Thr Asn Ser Asp Glu Ser Thr Lys Gly Phe Asp Asp Lys Glu Glu 615

Ser Glu Ile Thr Val Glu Cys Leu Asn Lys Arg Pro Asp Leu Lys 630

Glu Leu Val Arg Ser Glu Ile Lys Leu Ser Glu Leu Glu Asn Asn 645

Asn Ile Thr Phe Tyr Ser Cys Gly Pro Ala Thr Phe Asn Asp Asp 660

Phe Arg Asn Ala Val Val Gln Gly Ile Asp Ser Ser Leu Lys Ile 675

Asp Val Glu Leu Glu Glu Glu Ser Phe Thr Trp *** 687

SEQ ID No.: 3

Length : 17

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GACTCGAGTC GACATCG 17

SEQ ID No.: 4

Length : 24

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

ACACTTATTA GCACTTCATG TATT 24

SEQ ID No.: 5

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GAATTCTCTA GACTCCACCA TGGTTAGAAC CAGAGTCCTT TTCTGCCTCT TCATCTCTTT 60

CTTCGCTACA GTCCAATCGA GCG 83

SEQ ID No.: 6

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GTCCAATCGA GCGCTACACT CATCTCCACT TCATGCATTT CTCAGGCTGC ACTGTACCAG 60

TTCGGATGCT CAAGCAAGTC AAA 83

SEQ ID No.: 7

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

CAAGCAAGTC AAAGTCTTGC TACTGCAAGA ACATCAATTG GCTCGGAAGC GTCACTGCAT 60

GCGCTTATGA GAACTCCAAA TCT 83

SEQ ID No.: 8

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

TCCAGTGTGT AAACCTTGAT ACTTGAGCAT TGGCTGGCAA GTTTCATCAA AGCGGAGTCC 60

AGAGTCTTGT TAGATTTGGA GTT 83

SEQ ID No.: 9

Length : 83

Type   : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature   :

Name/Key : primer bind

SEQ :

TGTCTTCTTA TCGGATTTCT CAGGAGCGCG AAGGTAGTTA CTTGCATTAA GGTAGATGTT 60

CTTCATGTCC TCCAGTGTGT AAA 83

SEQ ID No.: 10

Length   : 83

Type   : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature   :

Name/Key : primer bind

SEQ :

GGATCCCATA GTTTTCCTCA TAGTAGTAGT GATAGGCCGT CTCATTTGCC ATCAACGGTT 60

GTGAAACAAC TGTCTTCTTA TCG 83

SEQ ID No.: 11

Length : 80

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GGATCCACTT GAATTTGATG CGATCTCAAT GGTGCGCATG GGGCCTCGTC TTCTTCTGGG 60

TCGCAGTCCT TACCGCCGCA 80

SEQ ID No.: 12

Length : 80

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

CCTTACCGCC GCAACTATCT TGAACATTCT CAAACGCGTA TTCGGCAAGA ACATTATGGC 60

AAATTCTGTT AAGAAGTCTC 80

SEQ ID No.: 13

Length : 80

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid
synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GTTAAGAAGT CTCTTATCTA CCCAAGCGTT TACAAAGACT ACAACGAGAG AACTTTCTAT 60

CTTTGGAAAC GTTTGCCATT 80

SEQ ID No.: 14

Length : 80

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid
synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

AGAGTGAGAG AATAGTCAGA ATGACAAAGA TAAGAACTAC GAGTCCTTTG CCTCGAGTTG 60

TAAAGTTGAA TGGCAAACGT 80

SEQ ID No.: 15

Length : 80

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

AATGCCATTG ATCTTCTCCA TCTAGGTCTA TCGTAAGGAT GTGGCAACTT GATGTTATGT 60

CCGAAAGAGA GTGAGAGAAT 80

SEQ ID No.: 16

Length : 80

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

TCCGGATACC GAAAAGGTAC ACCACGGGGA AAAGAGCGAT TGCCATCAAG TCAGCACGGC 60

GTGAGACGAA TGCCATTGAT 80

SEQ ID No.: 17

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

TCCGGAACAA CCCCTTCATC CCAATCACCG GATTGAGCTT TAGTACTTTC AACTTTTACC 60

ACAAATGGTC AGCATACGTC TGC 83

SEQ ID No.: 18

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GCATACGTCT GCTTCATGTT AGCCGTCGTC CATTCAATCG TTATGACCGC TTCAGGAGTT 60

AAACGAGGAG TATTCCAGTC TCT 83

SEQ ID No.: 19

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

TATTCCAGTC TCTTGTAAGG AAATTCTACT TCAGATGGGG AATAGTAGCC ACAATTCTTA 60

TGTCCATCAT CATTTTCCAG TCC 83

SEQ ID No.: 20

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

ATAAACATGA TGTTCATGGC TTTGTGAATA AGTAAGAAGA TTTCATAACC TCGGTTCCTG 60

AAGACCTTCT CGGACTGGAA AAT 83

SEQ ID No.: 21

Length : 83

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GAGGATGCCA GCCATGGACC AGATCCAGCC CATCCATCCT AGTGTGTGGC AATGGTAATA 60

CATAGCTATG ATAAACATGA TGT 83

SEQ ID No.: 22
Length : 83
Type : nucleic acid
Strandness: single
Topology : linear
Molecular type: other nucleic acid
synthetic DNA
Feature :
Name/Key : primer bind

SEQ :

GTCGACAAAG TGGCGGTCTT AAGACCTCCG TTCATGATGA TACGTACAAT TCGGCAGAAC 60

CTGTCGAAGC AGAGGATGCC AGC 83

SEQ ID No.: 23
Length : 82
Type : nucleic acid
Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GTCGACCACA GATGATTCTA ACGTTATCAA GATCTCTGTC AAGAAGCCTA AGTTCTTCAA 60

GTATCAAGTG GGAGCATTTG CC 82

SEQ ID No.: 24

Length : 82

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GGAGCATTTG CCTATATGTA CTTTCTTTCA CCAAAATCAG CCTGGTTCTA CAGTTTTCAA 60

TCTCATCCCT TCACAGTCCT AT 82

SEQ ID No.: 25

Length : 82

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

```
TTCACAGTCC TATCAGAAAG GCACAGAGAT CCTAACAACC CAGATCAACT AACTATGTAC   60

GTCAAAGCTA ACAAGGGCAT TA                                            82
```

SEQ ID No.: 26

Length : 82

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

```
CCTCTAAGAA AATCTTGCAA TCAACGGTAT GGTTTGGAGC GCTTAGAACT TTGCTAAGAA   60
```

GTACTCTCGT AATGCCCTTG TT 82

SEQ ID No.: 27

Length : 82

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GGCCCGCAGC TACTCCTACT AGATTTCTCT TAAGTTTGGC AATGTGAGGG ACAGTTACGC 60

CATATGGTCC CTCTAAGAAA AT 82

SEQ ID No.: 28

Length : 82

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

CTGCAGTTGA TCAGTGCTAG GCAATCTAAG GCATTCTACG AAATGGGGGT AGATGGCTGC 60

CACGCCGAGG CCCGCAGCTA CT 82

SEQ ID No.: 29
Length : 77
Type : nucleic acid
Strandness: single
Topology : linear
Molecular type: other nucleic acid
synthetic DNA
Feature :
Name/Key : primer bind

SEQ :

CTGCAGCACA AGTTCTACTG GATCGTCAAC GACCTTAGTC ACCTTAAGTG GTTCGAAAAC 60

GAGCTACAAT GGCTTAA 77

SEQ ID No.: 30
Length : 77
Type : nucleic acid
Strandness: single
Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

ACAATGGCTT AAGGAGAAAT CTTGTGAAGT CTCTGTCATC TACACTGGGT CATCAGTGGA 60

GGATACAAAC TCAGATG 77

SEQ ID No.: 31

Length : 77

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

CAAACTCAGA TGAGTCCACT AAGGGTTTCG ATGACAAGGA AGAATCTGAA ATCACCGTAG 60

AATGCCTTAA CAAGAGG 77

SEQ ID No.: 32

Length : 77

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

GTGATGTTGT TGTTCTCGAG TTCTGACAAT TTGATCTCTG ATCTCACTAG CTCTTTGAGG 60

TCTGGCCTCT TGTTAAG 77

SEQ ID No.: 33

Length : 77

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

CGATACCTTG TACAACTGCA TTCCTAAAGT CGTCATTGAA AGTCGCTGGT CCGCATGAGT 60

AGAAAGTGAT GTTGTTG 77

SEQ ID No.: 34

Length : 77

Type : nucleic acid

Strandness: single

Topology : linear

Molecular type: other nucleic acid

synthetic DNA

Feature :

Name/Key : primer bind

SEQ :

AAGCTTGAGC TCTTACCAAG TAAAACTCTC CTCCTCTAGT TCGACATCTA TCTTCAGACT 60

AGAATCGATA CCTTGTA 77